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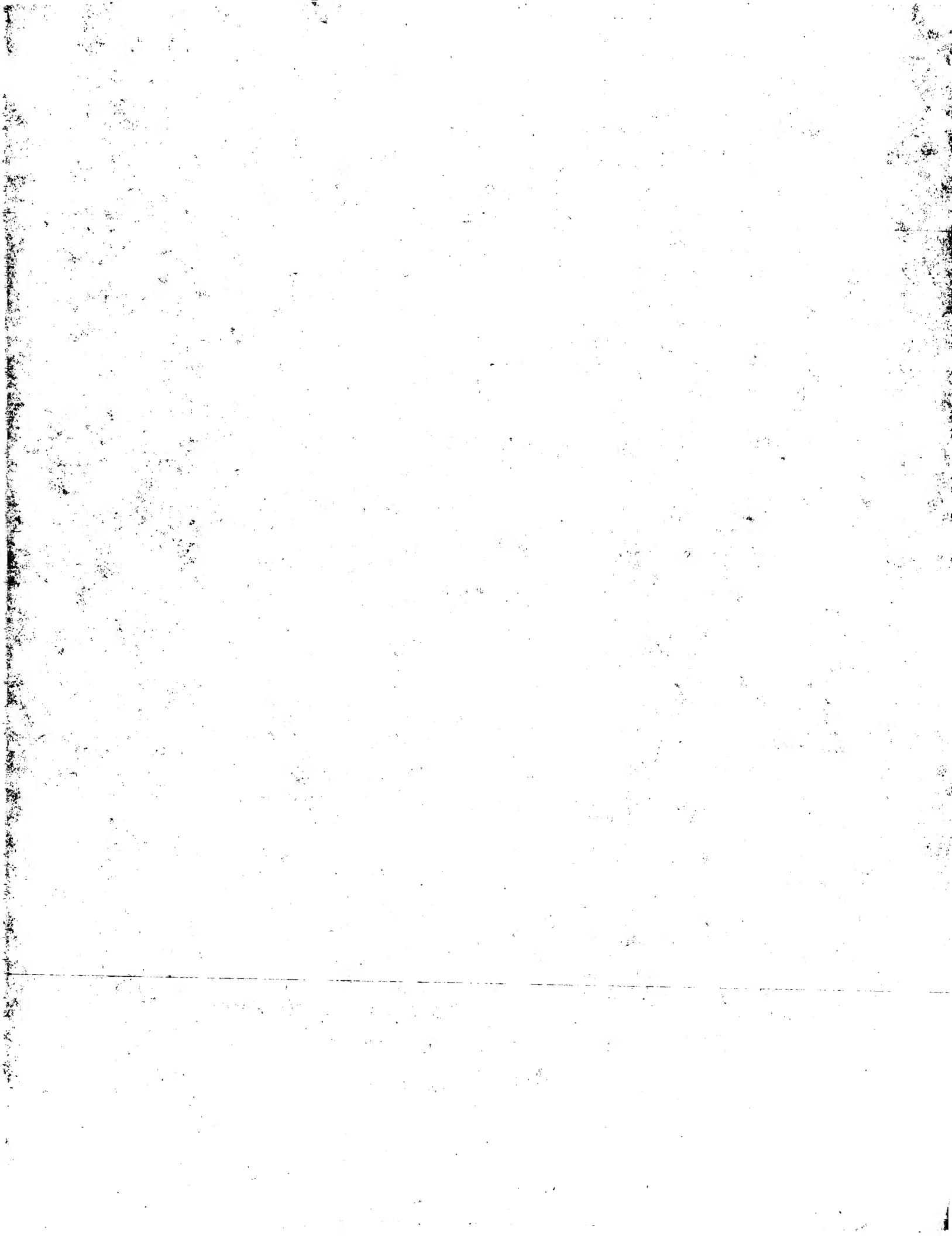
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US99/11023</p> <p>(22) International Filing Date: 18 May 1999 (18.05.99)</p> <p>(30) Priority Data: 09/080,625 18 May 1998 (18.05.98) US</p> <p>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/080,625 (CIP) Filed on 18 May 1998 (18.05.98)</p> <p>(71) Applicant (for all designated States except US): DEKALB GENETICS CORPORATION [US/US]; 3100 Sycamore Road, Dekalb, IL 60115-0600 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): KRIZ, Alan, L. [US/US]; 23 Robin Hood Drive, Gales Ferry, CT 06335 (US). SPENCER, T., Michael [US/US]; 11 Ivy Road, Mystic, CT 06335 (US).</p> <p>(74) Agent: HIGHLANDER, Steven, L.; Arnold White &amp; Durkee, P.O. Box 4433, Houston, TX 77210 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	
<p>(54) Title: METHODS AND COMPOSITIONS FOR TRANSGENE IDENTIFICATION</p> <p>(57) Abstract</p> <p>The present invention provides methods and compositions for the identification of transgenic seeds. This is accomplished by use of screenable markers linked to aleurone-specific promoters. The screenable markers can be provided as gene fusions with selectable markers, allowing both selection and screening of transformants. The use of aleurone-specific promoters, which also direct expression in embryogenic tissues, allows efficient selection of transgenic cells and the screening of viable transgenic seeds, while avoiding the deleterious effects associated with constitutive expression of screenable marker genes. Screening of transgenic seeds avoids the need for growing and assaying of seeds for transgenes and allows implementation of automated seed screening techniques for the identification of transgenic seeds.</p>			

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## DESCRIPTION OF THE INVENTION

### METHODS AND COMPOSITIONS FOR TRANSGENE IDENTIFICATION

#### BACKGROUND OF THE INVENTION

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##### **1. Field of the Invention**

The present invention relates generally to the field of agricultural biotechnology. More particularly, it concerns methods and compositions for the efficient identification of transgenic plant seeds.

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##### **2. Description of Related Art**

Many attempts have been made to genetically engineer desired traits into plant genomes by introduction of exogenous genes using genetic engineering techniques. The uptake of new DNA by recipient plant cells has been accomplished by various means, including *Agrobacterium* infection (Nester *et al.*, 1984), polyethylene glycol (PEG)-mediated DNA uptake (Lorz *et al.*, 1985), electroporation of protoplasts (Fromm *et al.*, 1986) and microprojectile bombardment (Klein *et al.*, 1987).

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An important aspect of the success achieved in transforming plants has been the ability to select or screen for transformed cells and/or plants. Most of the first successes in plant transformation relied on utilization of selectable markers for identification of transformed cells. Genes which have been used for selection of transformed plant cells include, for example, a neomycin phosphotransferase gene (Potrykus *et al.*, 1985) which provides resistance to kanamycin, paromomycin and G418; a *bar* gene which codes for bialaphos or phosphinothricine resistance (U.S. Patent No. 5,550,318); a mutant *aroA* gene which encodes an altered EPSP synthase protein conferring glyphosate resistance (Hinchee *et al.*, 1988); a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.*, 1988); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate resistant DHFR gene (Thillet *et al.*,

1988); a dalapon dehalogenase gene that confers resistance to the herbicide dalapon; and a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan.

5 More recently, interest has increased in utilization of screenable markers. One particularly useful screenable marker which has been discovered is the green fluorescent protein (GFP) (Sheen *et al.*, 1995). GFP was first cloned and sequenced from the cnidarian *Aequorea victoria* (Prasher *et al.*, 1992). Since then, it has been expressed in a variety of organisms, ranging from bacteria (Leff and Leff, 1996), fungi 10 (Spellig *et al.*, 1996), invertebrates (Plautz *et al.*, 1996), vertebrates (Zolotukhin *et al.*, 1996) and plants (Haseloff *et al.*, 1997; Reichel *et al.*, 1996; Sheen *et al.*, 1995; Tian *et al.*, 1997). An important advantage of GFP is that it can be assayed non-destructively. Firefly luciferase represents another useful fluorescent marker which can be assayed non-destructively, although detection requires addition of an 15 exogenous substrate (luciferin) for detection (Ow *et al.*, 1986; Millar *et al.*, 1995). In contrast, the assay of the screenable marker GUS is cytotoxic, and therefore, recovery of live transformed cells is difficult (Jefferson *et al.*, 1987).

One means that has been employed for the utilization of screenable markers, such as GFP, has been the fusion of the screenable marker with a selectable marker. Fusion proteins have been made, for example, between GFP and the selectable marker neomycin phosphotransferase (NPTII) (Genbank Accession No. AF004665), between GFP and hygromycin phosphotransferase (Lybarger *et al.* 1996), between the firefly luciferase gene (*luc*) and the neomycin phosphotransferase (NPTII) (Barnes, 1990), and between the ALS gene and GFP or GUS (WO 97/41228). The screening of transgenic maize cells based on GFP expression was described in WO 97/41228.

The identification of transgenic seeds using a GFP screenable marker also was described in WO 97/41228. In this case, however, the GFP gene was constitutively expressed using the ubiquitin promoter. It was found that seeds expressing GFP did not germinate when planted in soil, and could only be germinated by excision of embryos and plating on growth medium.

The failure to identify a method of non-destructively identifying transgenic seeds which can be directly germinated has represented a significant hindrance to breeders during the development of novel transgenic plant lines. Without labor-intensive embryo-rescue, current technology requires the growing and assaying of potentially transgenic seeds. The identification of a direct means for selection of transgenic cells, regeneration of plants from the transgenic cells and the screening of transgenic seeds which could, in turn, be directly advanced in breeding protocols would greatly improve scientists abilities to develop novel transgenic plant lines for the benefit of consumers.

### SUMMARY OF THE INVENTION

The current invention seeks to overcome deficiencies in the prior art by providing methods and composition for the efficient identification of transgenic seeds. Therefore, in one aspect, the current invention provides a construct comprising a screenable marker gene operably linked to an aleurone-specific promoter. This screenable marker gene can be provided as a gene fusion between the screenable marker gene and a selectable marker gene, wherein the gene fusion is operably linked to an aleurone-specific promoter. In particular embodiments of the invention, the screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene. In other embodiments of the invention, the selectable marker gene comprises a gene selected from the group consisting of NPTII, bar, EPSPS, anthranilate synthase and dalapon dehalogenase. In still other embodiments of the invention, the aleurone-specific promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, a barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine carboxypeptidaseII promoter, catalase promoter, alpha-glucosidase promoter, beta-amylase promoter, VP1 promoter, and bronze2 promoter. The oleosin promoter may be an L3 oleosin promoter. The marker gene constructs of the invention may additionally comprise first, second, third, or any

additional number of exogenous genes which can physically be placed on the construct. The exogenous gene may be operably linked to a second promoter.

In another aspect, the invention provides a transgenic plant comprising a screenable marker gene operably linked to an aleurone-specific promoter. This screenable marker gene can be provided as a gene fusion between a selectable marker gene and the screenable marker gene, wherein the gene fusion is operably linked to an aleurone-specific promoter. In one embodiment of the invention, the screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene. In other embodiments of the invention, the screenable marker is a GFP gene. The selectable marker gene may comprise any suitable gene, for example, bar, EPSPS, NPTII, anthranilate synthase or dalapon dehalogenase. The promoter may be selected from the group consisting of an L3 oleosin promoter, a globulin 1 promoter, and a barley LTP2 promoter. The plant may be a monocotyledonous plant, and may be further defined as a plant selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, sugarcane and turfgrass. In particular embodiments of the invention, the monocotyledonous plant is maize. The plant may also be a dicotyledonous plant, and may still further be selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco. In particular embodiments of the invention, the dicotyledonous plant is soybean.

In still yet another aspect, the invention provides a progeny plant of any generation of a transgenic plant comprising a screenable marker gene operably linked to an aleurone-specific promoter, wherein the progeny comprises said screenable marker gene. This screenable marker gene can be provided as a gene fusion between a selectable marker gene and the screenable marker gene, wherein the gene fusion is operably linked to an aleurone-specific promoter.

In still yet another aspect, the invention provides a method of identifying transgenic seeds comprising the steps of: (a) obtaining a transgenic plant, the cells of which comprise a screenable marker gene operably linked to an aleurone-specific promoter; (b) cultivating said plant until seed set; (c) collecting seeds produced on

5                   said plant; and (d) screening said seeds based on a phenotype conferred upon seeds by said screenable marker gene. In one embodiment of the invention, the screenable marker gene is provided as a gene fusion between a selectable marker gene and the screenable marker gene, wherein the gene fusion is operably linked to an aleurone-specific promoter. In particular embodiments of the invention, the screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene. The transgenic plant may be a monocotyledonous plant, and further, may be selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, sugarcane and turfgrass. In particular embodiments of the invention, the monocotyledonous plant is maize. The transgenic plant may also be a dicotyledonous plant, and further defined as selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco. In particular embodiments of the invention, the dicotyledonous plant is soybean.

15                   In the method of identifying seeds provided by the invention, the transgenic plant may further comprise an exogenous gene encoding a selected trait, wherein the exogenous gene is genetically linked to said screenable marker gene. The exogenous gene may still further comprise a promoter and 3' region operably linked to said exogenous gene. In particular embodiments of the invention, the exogenous gene encodes a trait selected from the group consisting of an insect resistance gene, a viral disease resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene. The screenable marker gene may also comprise a 3' region operatively linked to the gene, and also an element enhancing the expression of said gene. In particular embodiments of the invention, the screening of collected seeds is automated.

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In still yet another aspect, the invention provides a method of plant breeding comprising the steps of: (a) obtaining first and second plants, wherein cells of one or

both of said first or second plants comprises a screenable marker gene operably linked to an aleurone-specific promoter; (b) crossing said first and second plants; and (c) collecting the seeds resulting from said crossing. The method may further comprise the step of: (d) selecting transgenic seeds from said collected seeds based on a phenotype conferred upon said transgenic seeds by said screenable marker gene. The screenable marker gene can be provided as a gene fusion between a selectable marker gene and the screenable marker gene, wherein the gene fusion is operably linked to an aleurone-specific promoter. In particular embodiments of the invention, the screenable marker gene may be selected from the group consisting of a GFP gene, a luciferase gene, and an R gene. In other embodiments of the invention, the selectable marker gene is selected from the group consisting of bar, EPSPS, NPTII, anthranilate synthase and dalapon dehalogenase. In still further embodiments of the invention, the aleurone-specific promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine carboxypeptidaseII promoter, catalase promoter, alpha-glucosidase promoter, beta-amylase promoter, VP1 promoter, and bronze2 promoter. Where the promoter is an oleosin promoter, an L3 oleosin promoter may be used.

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In the method of plant breeding, the first and second transgenic plants may be monocotyledonous plants, and may be selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, sugarcane and turfgrass. In particular embodiments of the invention, the monocotyledonous plants are maize plants. The plants may also be dicotyledonous plants, and can be selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco. In one embodiment of the invention, the dicotyledonous plants are soybean plants. In other embodiments of the invention, the step of selecting can be automated.

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In still yet another aspect, the invention provides a method of plant breeding comprising the steps of: (a) obtaining a transgenic plant, the cells of which comprise a screenable marker gene operably linked to an aleurone-specific promoter; (b)

allowing said plant to self fertilize; and (c) collecting the seeds produced on said plant. The method may further comprise the step of: (d) selecting transgenic seeds from said collected seeds based on the phenotype conferred upon said transgenic seeds by said screenable marker gene. The screenable marker gene can be provided as a gene fusion between a selectable marker gene and the screenable marker gene, wherein the gene fusion is operably linked to an aleurone-specific promoter. In particular embodiments of the invention, the screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene. In other embodiments of the invention, the selectable marker gene is selected from the group consisting of bar, EPSPS, NPTII, anthranilate synthase and dalapon dehalogenase. In still further embodiments of the invention, the aleurone-specific promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, a barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine carboxypeptidaseII promoter, catalase promoter, alpha-glucosidase promoter, beta-amylase promoter, VP1 promoter, and bronze2 promoter. In certain embodiments of the invention, the oleosin promoter may be an L3 oleosin promoter. comprises a globulin 1 promoter. The transgenic plant may be a monocotyledonous plant, and may still further be selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, sugarcane and turfgrass. In particular embodiments of the invention, the monocotyledonous plant is a maize plant. The transgenic plant may also be a dicotyledonous plant, and may further be selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco. In particular embodiments of the invention, the dicotyledonous plant is a soybean plant. In other embodiments of the invention, the step of selecting may be automated.

BRIEF DESCRIPTION OF THE DRAWINGS

5 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

10 **FIG. 1:** Map of plasmid DV130. The 6237 bp plasmid contains an expression cassette comprising a 1039 bp promoter from the maize L3 oleosin gene; the coding sequence of a MGFP::NPTII translational fusion; and the Tr7 terminator.

15 **FIG. 2:** Map of plasmid DV131. The 6054 bp plasmid contains an expression cassette comprised of a 1039 bp promoter from the maize L3 oleosin gene; the coding sequence of a EGFP::NPTII translational fusion; and the Tr7 terminator.

20 **FIG. 3:** Map of plasmid DV132. The 6778 bp plasmid contains an expression cassette comprised of a 1039 bp promoter from the maize L3 oleosin gene; the rice actin 1 intron 1; the coding sequence of a MGFP::NPTII translational fusion; and the Tr7 terminator.

25 **FIG. 4:** Map of plasmid DV133. The 6595 bp plasmid contains an expression cassette comprised of a 1039 bp promoter from the maize L3 oleosin gene; the rice actin 1 intron 1; the coding sequence of a EGFP::NPTII translational fusion; and the Tr7 terminator.

**FIG. 5:** Map of plasmid PBSK/EGFP/NPTII. The 4530 bp plasmid contains the coding sequence of a EGFP::NPTII translational fusion inserted in-frame into the beta-galactosidase protein coding sequence in the pBSK plasmid.

30 **FIG. 6:** Map of plasmid PBSK/MGFP/NPTII. The 4690 bp plasmid construct contains the coding sequence of a MGFP::NPTII translational fusion inserted in-frame into the beta-galactosidase protein coding sequence in the pBSK plasmid.

30 DETAILED DESCRIPTION OF THE INVENTION

The current invention overcomes deficiencies in the prior art by providing constructs which allow the visual identification of seeds which have inherited a

transgene prepared in accordance to the instant invention. In particular, the present invention provides transformation constructs which include a screenable marker which is preferentially expressed in the seed coat or aleurone of seeds, but not in the whole seed. Such promoters also direct expression of the construct in embryogenic tissues, thereby allowing the identification and regeneration of transgenic cells into whole plants. The expression of the screenable marker gene specifically in embryogenic tissues and the aleurone allows for direct detection of transgenic cells and seeds, while avoiding the deleterious effects associated with the expression of green fluorescent protein (GFP) and other screenable markers in whole seeds or plants.

The constructs of the invention also are provided as gene fusions between selectable and screenable marker genes. Gene fusions are expressed as a single unit, yet produce a gene product conferring both the selectable and screenable marker gene phenotypes of the non-fused starting genes. Because the genes are expressed together, expression of the selectable marker phenotype is directly correlated with the screenable marker phenotype. In cases where selectable and screenable marker genes are transformed on different DNA segments, or even on the same DNA segment but under the control of a separate promoter, the expression of one marker may often not be correlated with the expression of the other.

By expression of the selectable-screenable marker gene fusion with a promoter which directs expression specifically in the aleurone, but also in embryogenic cells, transgenic cells can be identified using a selective agent and transgenic seeds identified by expression of the screenable marker. Selection of transgenic cells is often preferable to screening, because typically only a small number of target cells will be transformed. In cases where intact cells are transformed, screening for transgenic cells can lead to regeneration of chimeric plants, wherein regenerated plants contain both transgenic and non-transgenic cells. The tissue-specific expression of a selectable-screenable marker gene fusion is important because it serves to eliminate problems associated with constitutive expression of selectable marker genes, and in particular, with GFP.

Preferably, the DNA construct which comprises the screenable marker gene also contains at least a first exogenous gene encoding a selected trait. Alternatively, a construct comprising an exogenous gene may be co-transformed with a second 5 construct comprising a screenable marker in accordance with the current invention. In these instances, the screenable marker or selectable-screenable marker gene phenotype typically will be genetically linked to the exogenous gene and, therefore, can be used to predict the presence of the exogenous gene. As such, a screenable marker gene expressed, for example, in a seed, can provide predictive value for the presence of 10 exogenous genes which otherwise could not be non-destructively assayed at the whole-seed level. The exogenous genes used in combination with the marker genes of the invention may confer potentially any trait one desires to have expressed in a transgenic plant. Specific examples of such traits and the genes which confer these traits are disclosed herein below.

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### I. Marker Gene Compositions

The present invention provides methods and composition for the expression and identification of screenable marker genes in seeds, thereby allowing rapid visual confirmation of transgenic seeds. While the expression constructs of the invention 20 could include only a screenable marker gene linked to an aleurone-specific promoter, more preferred will be constructs which also include a selectable marker gene. The selectable marker allows for efficient isolation of transformed cells and subsequent regeneration of transformed plants, which would typically not be achieved using screening techniques alone. Even more preferred for use will be a screenable marker 25 gene which has been fused to a selectable marker gene to form a selectable-screenable marker gene fusion.

The protein produced by a selectable-screenable marker gene fusion exhibits the activity of both the selectable and the screenable marker genes, yet comprises a 30 single transcribed gene product, thereby decreasing the overall size of the construct and minimizing size constraints for the inclusion in the construct of one or more expressible genes encoding a selected trait. Additionally, by decreasing the overall

size of the construct used for transformation, the likelihood of obtaining transgenes with rearranged insertion events is minimized. Still further, utilization of a fusion protein should maximize the correlation between expression of the marker genes with the presence of any exogenous genes encoded on the construct.

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A number of different screenable and selectable marker genes are known in the art and could be employed for the preparation of a selectable-screenable marker gene fusion. For use with the instant invention, the screenable marker gene should be detectable when expressed in seeds using non-lethal assays and should be 10 preferentially expressed in the aleurone or embryo. The selectable marker gene should allow for efficient recovery of transformed cells and subsequent recovery of transformed plants. The selectable and screenable marker genes also should be capable of forming a fusion protein which exhibits the activities of both marker genes. Further, it is preferable that marker genes having shorter overall gene sequences be 15 used in order to limit the length of the resulting construct.

One particularly useful screenable marker for the production of selectable-screenable marker gene fusions, in accordance with the current invention, is the green fluorescent protein (GFP) (Sheen *et al.*, 1995). Detection of GFP fluorescence does not 20 require a cofactor or a substrate, and is non-lethal (Cody *et al.*, 1993). Detection of GFP in living cells only requires excitation by light at 395 or 470 nm. Additionally, many variants of GFP have been produced which exhibit various desirable characteristics such as altered wavelengths of fluorescence excitation and emission.

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Another screenable marker gene contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene (Ow *et al.*, 1986). The presence of the *lux* gene is detected by immersion of the transformed cells in the substrate luciferin, followed by detection using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell 30 luminometry. Genes from the maize R gene complex also could be used as screenable markers with the current invention. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant

tissue. Maize strains can have one, or as many as four, R alleles which combine to regulate pigmentation in a developmental and tissue specific manner. Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant alleles for genes encoding for the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, A1, A2, Bz1 and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment formation. Exemplary lines include Wisconsin 22 which contains the rg-Stadler allele and TR112, a K55 derivative which is r-g, b, Pl. Alternatively, any plant species can be utilized if the C1 and R alleles are introduced together.

A number of selectable marker genes also may be used in connection with the present invention. One selectable marker gene deemed especially useful for the creation of selectable-screenable marker gene fusions in accordance with the instant invention is the neomycin phosphotransferase II (NPTII) gene (Potrykus *et al.*, 1985) which codes for kanamycin resistance and can be selected for using kanamycin, G418, paromomycin, *etc.* Examples of other selectable marker genes which could potentially be used with the instant invention include a *bar* gene from *Streptomyces hygroscopicus* or a *pat* gene from *Streptomyces viridochromogenes*, which genes confer bialaphos resistance; a mutant EPSP synthase gene (Hinchee *et al.*, 1988) conferring glyphosate resistance; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.*, 1988); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (European Patent Application 154,204); a methotrexate resistant DHFR gene (Thillet *et al.*, 1988); a dalapon dehalogenase gene that confers resistance to the herbicide dalapon; a hygromycin phosphotransferase gene conferring resistance to hygromycin or a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan. Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, *e.g.*, CTP (U.S. Patent No. 5,188,642) or OTP (U.S. Patent No. 5,633,448) and use of the modified maize EPSPS gene described in PCT Application WO 97/04103.

## II. Optimization of GFP

Various modifications may be made in the gene encoding green fluorescent protein to modify its characteristics as a fluorescent marker. These modifications are specifically contemplated by the inventors for use with the current invention. In particular, it is contemplated that the utility of GFP for the identification of transformed seeds may be supplemented by the development of chromophore-mutated versions of GFP, in which amino acid substitutions have produced forms of the protein with shifted excitation peaks, increased stability, single excitation and emission peaks, brighter fluorescence, and more rapid chromophore formation (Heim *et al.*, 1995). These modifications can be used to increase the sensitivity of GFP detection, allow simultaneous analysis of two GFP constructs per cell, seed or organism, and offer better matching to standard filter sets.

Wild-type GFP has two excitation points at about 395 and 470 nm, and it emits the most amount of green light at 508–509 nm (Chalfie *et al.*, 1994a). A blue-shifted variant can be produced by substituting histidine or tryptophan for tyrosine at position 66 in the chromophore (Heim and Tsien, 1996). These mutants, called blue fluorescent proteins (BFPs) (Heim and Tsien, 1996) were created to enhance GFP as a molecular tool by providing a second visibly distinct color for *in vivo* visualization of subcellular activities (Heim *et al.*, 1994). Similarly a GFP protein has been produced with a red-shifted excitation peak (Delagrave *et al.*, 1995).

Other mutations, made by changing a serine at amino acid 65 to cysteine and threonine, have increased the magnitude of green fluorescence, significantly improving the detection levels of GFP (Reichel *et al.*, 1996). In addition, other modifications, known as F64L and S65T, have improved the folding in a marker known as EGFP, a modified version of the native GFP protein (Cormack *et al.*, 1996; Yang *et al.*, 1996a).

Though the wild-type gene has been expressed in both monocot and dicot cells (Reichel *et al.*, 1996), expression had generally been low or unstable in plants (Hu and

Cheng, 1995). Therefore, modified versions of the gene, such as *mgfp4*, were produced when an 84-bp sequence necessary for proper protein folding and expression was found to be mis-spliced in *Arabidopsis* (Haseloff and Amos, 1995). The sequence similarity to known plant introns caused it to be spliced-out during transcription, so Haseloff and colleagues reduced its AT content and reduced the chance of the sequence being excised, thereby restoring proper expression in the plant (Haseloff *et al.*, 1997). To help improve visible fluorescence from GFP, a modified GFP construct (*mgfp5-er*) has also been produced that targets the protein to the endoplasmic reticulum, resulting in high, *in vivo*, expression in plants (Haseloff *et al.*, 10 1997).

### III. Codon Modifications for Plant Gene Expression

It may, in particular embodiments of the invention, be desirable to utilize genes in which the native DNA sequence has been modified to enhance expression in maize or other plants. Specifically, it may be desired to adjust the DNA sequence of the transgene to more closely resemble that of genes which are efficiently expressed in the host plant. For example, it has been shown that by modifying the codon usage of the GFP gene to decrease the overall AT content of the gene, expression of the GFP protein can be dramatically increased in corn protoplasts and tobacco plants (Chiu *et* 15 20 *al.*, 1996).

Such codon modifications may be carried out by substitution of bases in a native gene sequence without changing the sequence of the encoded polypeptide. In this manner, an identical polypeptide may be produced, but at levels much higher than 25 would otherwise be produced using constructs comprising the native gene sequence. Such alterations may be particularly desirable in the case of genes which were originally isolated from organisms distantly related to the plant host, such as with the case of bacterial genes.

30 In designing synthetic genes for enhanced expression in plants, the DNA sequence of the native structural gene is modified in order to contain codons preferred by highly expressed plant genes. Preferably, the A+T content of the synthetic gene is

substantially equal to that of genes for highly expressed proteins in the host plant. In genes encoding highly expressed plant proteins, the A+T content is approximately 55%. It is preferred that the synthetic gene have an A+T content near this value, and not sufficiently high as to cause destabilization of RNA and, therefore, lower the protein expression levels. More preferably, the A+T content is no more than about 5 60% and most preferably is about 55%.

It is known in the art that seldom used codons are generally detrimental to gene expression and must be avoided or used judiciously. Thus, in designing a 10 synthetic gene encoding the desired transgene polypeptide, individual amino acid codons found in the original gene are altered to reflect the codons preferred by the host plant for a particular amino acid. However, attention is given to maintaining the overall distribution of codons for each amino acid within the coding region of the gene. Hence, the synthetic gene is designed such that its distribution frequency of 15 codon usage deviates, preferably, no more than 25% from that of highly expressed plant genes and, more preferably, no more than about 10%. Preferred codons used in maize, for example, are given in Table 1. The preferred codons for design of synthetic genes for expression in other plants will be well known to those of skill in the art and may be found in, for example, Wada *et al.*, 1990.

20 In designing a heterologous gene for expression in plants, sequences which interfere with the efficacy of gene expression, such as plant polyadenylation signals, polymerase II termination sequences, hairpins, plant consensus splice sites and the like, are eliminated. Also, for ultimate expression in plants, the synthetic gene 25 nucleotide sequence is preferably modified to form a plant initiation sequence at the 5' end of the coding region. In addition, particular attention is preferably given to assure that unique restriction sites are placed in strategic positions to allow efficient assembly of oligonucleotide segments during construction of the synthetic gene and to facilitate subsequent nucleotide modification. Consideration will also be given to the 30 percentage G+C content of the degenerate third base (monocot plants appear to favor G+C in this position, whereas dicots do not). It is recognized that the XCG nucleotide is the least preferred codon in dicots whereas the XTA codon is avoided in both

monocots and dicots. Synthetic genes will also preferably have CG and TA doublet avoidance indices approximating those of the chosen host plant.

As is known to those skilled in the art of synthesizing genes, the DNA sequence to be synthesized is divided into segment lengths which can be synthesized conveniently and without undue complication. Each segment has unique restriction sequences at the cohesive ends. Single-stranded oligonucleotides are annealed and ligated to form the DNA segments. The length of the protruding cohesive ends in complementary oligonucleotide segments is four to five residues. In the strategy evolved for gene synthesis, the sites designed for the joining of oligonucleotide pieces and DNA segments are different from the restriction sites created in the gene. The nucleotide sequence of each fragment is determined at this stage by the dideoxy method using recombinant phage DNA as templates and selected synthetic oligonucleotides as primers. Each segment is individually excised at the flanking restriction sites from its cloning vector and spliced into the vector containing segment. Most often, segments are added as a paired segment, instead of as a single segment, to increase efficiency. In this way, the entire gene is constructed in the original plasmid. Exemplary procedures for the design and production of synthetic genes which may be used in the context of the current invention are disclosed in, for example, U.S. Patent No. 5,567,600; U.S. Patent No. 5,567,862; U.S. Patent No. 5,500,365; and U.S. Patent No. 5,508,468, each of which is specifically incorporated herein by reference in its entirety.

Table 1: Preferred Maize DNA Codons

Amino Acids		Codons (most preferred→less preferred)					
Alanine	Ala	A	GCC	GCT	GCG	GCA	
Cysteine	Cys	C	TGC	TGT			
Aspartic acid	Asp	D	GAC	GAT			
Glutamic acid	Glu	E	GAG	GAA			
Phenylalanine	Phe	F	TTC	TTT			
Glycine	Gly	G	GGC	GGT	GGG	GGA	
Histidine	His	H	CAC	CAT			
Isoleucine	Ile	I	ATC	ATT	ATA		
Lysine	Lys	K	AAG	AAA			
Leucine	Leu	L	CTG	CTC	CTT	TTG	CTA TTA
Methionine	Met	M	ATG				
Asparagine	Asn	N	AAC	AAT			
Proline	Pro	P	CCG	CCA	CCC	CCT	
Glutamine	Gln	Q	CAG	CAA			
Arginine	Arg	R	CGC	AGG	CGG	CGT	AGA CGA
Serine	Ser	S	AGC	TCC	TCG	TCT	TCA AGT
Threonine	Thr	T	ACC	ACG	ACT	ACA	
Valine	Val	V	GTG	GTC	GTT	GTA	
Tryptophan	Trp	W	TGG				
Tyrosine	Tyr	Y	TAC	TAT			

#### IV. Automated Screening of Transgenic Seeds

5       Automated screening techniques may be implemented with the current invention for the identification of transgenic seeds. By implementation of automated techniques, large numbers of seeds can be efficiently screened and the transgenic seeds among a total population of seeds collected. Automated techniques may be faster, less expensive and more accurate than reliance upon human technicians.

10

Seed sorting machines which could be used with the current invention have been described. For example, described in U.S. Patent No. 4,946,046, the disclosure of which is specifically incorporated herein by reference in its entirety, is an apparatus for sorting seeds according to color. In this machine, seeds are sorted according to color by placing the seeds in uniform rows of indentations in a rotating drum and passing the seeds beneath a digital imaging camera and a light source. Images are read by the camera and are fed to a computer, which also receives information from a drum speed sensor. The computer generates a signal which causes a blast of air to

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blow through an opening in the bottom of an indentation containing a colored seed to collect such seed. Collected seeds are fed into a collection hopper, and the non-colored seeds into a separate hopper.

5 By varying the wavelength of the light source used for detection of colored seeds, as well as barrier filters placed between the colored seed and the detection camera, potentially any screenable marker could be detected with this technique. For example, to detect seeds expressing GFP, the excitation wavelength is in the blue light - UV spectrum, typically at about 395 nm. Suitable light sources for UV emission are well known to those of skill in the art, and include xenon or mercury lamps. Suitable filter sets also are well known to those of skill in the art, and include, for example, a BP450-490 exciter filter, an FT510 chromatic beam splitter, and a BP515-565 barrier filter (Carl Zeiss, Inc., Thornwood, NY). Such filter sets and emission wavelengths are discussed in more detail in Heim and Tsien, 1996, the disclosure of which is 10 15 specifically incorporated herein by reference in its entirety.

The detection system described above also may be adapted to sort seeds based on the strength of coloration of the seeds. By decreasing the intensity of the light source, or changing the selection criteria, only those seeds which strongly express the 20 screenable marker gene will be selected. It is specifically contemplated that this will allow the selection of highly expressing transformation events. Further, by use for transformation of constructs comprising one or more exogenous genes in addition to selectable-screenable marker genes, the selective power can be extended to the exogenous genes. Therefore, large numbers of transgenic seeds, representing a variety 25 of different transformation events, can be efficiently screened and only those transformation events which are highly expressed selected.

## V. Transformation Constructs

In addition to marker genes, the DNA constructs of the invention may further 30 include structures such as exogenous genes, enhancers, polylinkers, or regulatory genes as desired. The DNA constructs may be in the form of vectors and plasmids, or linear DNA fragments. Whole vector DNA may be used for transformation, or

alternatively, an isolated expression cassette containing the constructs of the invention can be used. The constructs may additionally be co-transformed into recipient cells with one or more other vectors. Using this method, it is assumed that the additional vector inserts into the host genome at a location which is genetically linked to the marker gene, thereby allowing the use of the marker gene for identification of transformants which include the co-transformed vector DNA. The constructs of the invention used for transformation may be contained on vectors such as plasmids, cosmids, YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes) or DNA segments isolated therefrom.

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(i) *Regulatory Elements*

The constructs of the invention will include a selectable-screenable marker gene fusion, wherein the screenable marker permits the identification of seeds which have inherited the transgene construct. As such, the marker gene fusion should be operably linked to a promoter capable of preferentially directing expression in seeds. Where a selectable-screenable marker gene fusion is used, the promoter will also preferably be expressed in the target tissue which is used for transformation, thereby allowing efficient selection of transformed cells. For example, callus or embryo expression of the selectable-screenable marker gene fusion would allow utilization of a selection agent for efficient recovery of transformed cells. The promoter should also not direct expression of the marker gene in the whole plant and seed, as it has been reported that some screenable marker genes may exhibit phytotoxicity. Examples of the most preferred promoters for use with the instant invention include the maize L3 oleosin, maize globulin 1 and barley LTP 2 promoters. Examples of some other suitable promoters for directing the expression of the constructs of the invention include the alpha-amylase, chitinase, beta-glucanase, cysteine proteinase, glutaredoxin, HVA1, serine carboxypeptidaseII, catalase, alpha-glucosidase, beta-amylase, VP1, and bronze2 promoters, as well as oleosin promoters in addition to the L3 oleosin promoter.

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As indicated above, the constructs of the invention will preferably include, in addition to a screenable marker gene or selectable-screenable marker gene fusion, at

least a first exogenous gene. For expression of exogenous genes, virtually any promoter capable of directing gene expression in plants can be used. Which promoter is used will depend on the gene being expressed and the desired pattern of expression, and will be known to those of skill in the art in light of the instant disclosure. Useful 5 promoters for the expression of an exogenous gene include those that are inducible, viral, synthetic, constitutive, temporally regulated, spatially regulated, and spatio-temporally regulated (Chau *et al.*, 1989; Poszkowski *et al.*, 1989; Odell *et al.*, 1985).

10 A promoter is selected for its ability to direct the transformed plant cell's or transgenic plant's transcriptional activity to the coding region. Promoters can be near-constitutive, such as the CaMV 35S promoter (Odell *et al.*, 1985), CaMV 19S (Lawton *et al.*, 1987), *nos* (Ebert *et al.*, 1987), *Adh* (Walker *et al.*, 1987), sucrose synthase (Yang & Russell, 1990),  $\alpha$ -tubulin, actin (Wang *et al.*, 1992), *cab* (Sullivan *et al.*, 1989), PEPCase (Hudspeth & Grula, 1989) and R gene complex-associated 15 promoters (Chandler *et al.*, 1989). Where the promoter is a near-constitutive promoter, increases in polypeptide expression generally are found in a variety of transformed plant tissues (e.g., callus, leaf, seed and root).

20 Alternatively, expression of transgenes can be directed to specific plant tissues by using vectors containing a tissue-specific promoter. An exemplary tissue-specific promoter is the lectin promoter (Vodkin *et al.*, 1983; Lindstrom *et al.*, 1990.). Other exemplary tissue-specific promoters are corn sucrose synthetase 1 (Yang *et al.*, 1990), corn alcohol dehydrogenase 1 (Vogel *et al.*, 1989), corn light harvesting complex 25 (Simpson, 1986), corn heat shock protein (Odell *et al.*, 1985), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (Langridge *et al.*, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 30 1 (Keller *et al.*, 1989), truncated CaMV 35s (Odell *et al.*, 1985), Potato patatin promoters (Wenzler *et al.*, 1989), maize zein and globulin-1 promoters. Other tissue specific promoters including root cell promoters (Conkling *et al.*, 1990) and tissue specific enhancers (Fromm *et al.*, 1989) also are contemplated to be particularly useful, as are inducible promoters such as ABA- and turgor-inducible promoters.

Ultimately, the most desirable DNA segments for introduction into a monocot genome may be homologous genes or gene families which encode a desired trait (for example, increased yield per acre), and which are introduced under the control of novel promoters or enhancers, *etc.*, or perhaps even homologous or tissue specific (e.g., root-, collar/sheath-, whorl-, stalk-, earshank-, kernel- or leaf-specific) promoters or control elements. Therefore, when the term "exogenous gene" is used, is it intended to include genes which are native to the host genome, but which are under the control of a novel regulatory element. Indeed, it is envisioned that a particular use of the present invention may be the production of transformants comprising a transgene which is targeted in a tissue-specific manner. For example, insect resistant genes may be expressed specifically in the whorl and collar/sheath tissues which are targets for the first and second broods, respectively, of European Corn Borer (ECB). Likewise, genes encoding proteins with particular activity against rootworm may be targeted directly to root tissues.

Vectors for use in tissue-specific targeting of gene expression in transgenic plants typically will include tissue-specific promoters and also may include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure.

It also is contemplated that tissue specific expression may be functionally accomplished by introducing a constitutively expressed gene (all tissues) in combination with an antisense gene that is expressed only in those tissues where the gene product is not desired. For example, a gene coding for the crystal toxin protein from *B. thuringiensis* (Bt) may be introduced such that it is expressed in all tissues using the 35S promoter from Cauliflower Mosaic Virus. Alternatively, a rice actin promoter or a histone promoter from a dicot or monocot species also could be used for constitutive expression of a gene. Furthermore, it is contemplated that promoters combining elements from more than one promoter may be useful. For example, U.S. Patent No. 5,491,288 discloses combining a Cauliflower Mosaic Virus promoter with

a histone promoter. Expression of an antisense transcript of the Bt gene in a maize kernel, using for example a zein promoter, would prevent accumulation of the Bt protein in seed. Hence the protein encoded by the introduced gene would be present in all tissues except the kernel. It is specifically contemplated by the inventors that a similar strategy could be used with the instant invention to direct expression of a screenable marker in seed tissue.

Alternatively, one may wish to obtain novel tissue-specific promoter sequences for use in accordance with the present invention. To achieve this, one may 10 first isolate cDNA clones from the tissue concerned and identify those clones which are expressed specifically in that tissue, for example, using Northern blotting. Ideally, one would like to identify a gene that is not present in a high copy number, but which gene product is relatively abundant in specific tissues. The promoter and control elements of corresponding genomic clones may then be localized using the techniques 15 of molecular biology known to those of skill in the art.

It is contemplated that expression of some genes in transgenic plants will be desired only under specified conditions. For example, it is proposed that expression 20 of certain genes that confer resistance to environmental stress factors such as drought will be desired only under actual stress conditions. It further is contemplated that expression of such genes throughout a plants development may have detrimental effects. It is known that a large number of genes exist that respond to the environment. For example, expression of some genes such as *rbcS*, encoding the 25 small subunit of ribulose bisphosphate carboxylase, are regulated by light as mediated through phytochrome. Other genes are induced by secondary stimuli. For example, synthesis of abscisic acid (ABA) is induced by certain environmental factors, including but not limited to water stress. A number of genes have been shown to be induced by ABA (Skriver and Mundy, 1990). It also is anticipated that expression of 30 genes conferring resistance to insect predation would be desired only under conditions of actual insect infestation. Therefore, for some desired traits, inducible expression of genes in transgenic plants will be desired.

It is proposed that, in some embodiments of the present invention, expression of a gene in a transgenic plant will be desired only in a certain time period during the development of the plant. Developmental timing frequently is correlated with tissue specific gene expression. For example, expression of zein storage proteins is initiated 5 in the endosperm about 15 days after pollination.

It also is contemplated that it may be useful to target DNA itself within a cell. For example, it may be useful to target introduced DNA to the nucleus as this may increase the frequency of transformation. Within the nucleus itself it would be useful 10 to target a gene in order to achieve site specific integration. For example, it would be useful to have a gene introduced through transformation replace an existing gene in the cell.

#### 1. Untranslated Leader Sequences

Another type of element which can regulate gene expression is the DNA 15 sequence between the transcription initiation site and the start of the coding sequence, termed the untranslated leader sequence. The leader sequence can influence gene expression and compilations of leader sequences have been made to predict optimum or sub-optimum sequences and generate "consensus" and preferred leader sequences 20 (Joshi, 1987). Preferred leader sequences are contemplated to include those which comprise sequences predicted to direct optimum expression of the attached gene, *i.e.*, to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. 25 Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, will be most preferred.

#### 2. Enhancers

Transcription enhancers or duplications of enhancers could be used to increase 30 expression. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. In some instances these 5'

enhancing elements are introns. Examples of enhancers include elements from the CaMV 35S promoter, octopine synthase genes (Ellis *et al.*, 1987), the rice actin 1 gene, the maize alcohol dehydrogenase gene, the maize shrunken 1 gene and promoters from non-plant eukaryotes (*e.g.* yeast; Ma *et al.*, 1988).

5

Specifically contemplated for use in accordance with the present invention are vectors which include the ocs enhancer element. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (ocs) gene of *Agrobacterium* (Ellis *et al.*, 1987), and is present in at least 10 other promoters (Bouchez *et al.*, 1989).

10

It is proposed that the use of an enhancer element, such as the ocs element and particularly multiple copies of the element, may be used to increase the level of transcription from adjacent promoters when applied in the context of monocot transformation.

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## (ii) *Terminators*

Constructs will typically include the gene of interest along with a 3' end DNA sequence that acts as a signal to terminate transcription and allow for the polyadenylation of the resultant mRNA. The most preferred 3' elements are contemplated to be those from the nopaline synthase gene of *Agrobacterium tumefaciens* (nos 3' end) (Bevan *et al.*, 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as Adh intron 1 (Callis *et al.*, 1987), sucrose synthase intron (Vasil *et al.*, 1989) or TMV omega element (Gallie, *et al.*, 1989), may further be included where desired.

25

(iii) *Transit or signal peptides*

Sequences that are joined to the coding sequence of a gene, which are removed post-translationally from the initial translation product and which facilitate the transport of the protein into or through intracellular or extracellular membranes are termed transit (usually into vacuoles, vesicles, plastids and other intracellular organelles) and signal sequences (usually to the outside of the cellular membrane). By facilitating the transport of the protein into compartments inside and outside the cell

these sequences may increase the accumulation of gene product protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of the gene may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. It is further contemplated that targeting of certain proteins may be desirable in order to enhance the stability of the protein (U.S. Patent No. 5,545,818, incorporated herein by reference in its entirety).

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This generally will be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, *e.g.*, vacuole, vesicle, plastid and mitochondrial membranes, whereas signal peptides direct proteins through the extracellular membrane.

A particular example of such a use concerns the direction of a protein conferring herbicide resistance, such as the mutant EPSPS protein, to a particular organelle such as the chloroplast rather than to the cytoplasm. This is exemplified by the use of the rbcS transit peptide, the chloroplast transit peptide described in European Patent No. 0189707, or the optimized transit peptide described in U.S. Patent No. 5,510,471, which confers plastid-specific targeting of proteins. In addition, it may be desirable to target certain genes responsible for male sterility to the mitochondria, or to target certain genes for resistance to phytopathogenic organisms to

the extracellular spaces, or to target proteins to the vacuole. A further use concerns the direction of enzymes involved in amino acid biosynthesis or oil synthesis to the plastid. Such enzymes include dihydrodipicolinic acid synthase which may contribute to increasing lysine content of a feed.

5

#### VI. Exogenous Genes for Modification of Plants

A particularly important advance of the present invention is that it provides methods and compositions for the efficient identification of plant seeds comprising transgene constructs having exogenous genes in addition to marker genes. Such exogenous genes will preferably confer the host plant with a desirable phenotype and often will be genes that direct the expression of a particular protein or polypeptide product, but they also may be non-expressible DNA segments, *e.g.*, transposons such as Ds that do not direct their own transposition. As used herein, an "expressible gene" is any gene that is capable of being transcribed into RNA (*e.g.*, mRNA, antisense RNA, *etc.*) or translated into a protein, expressed as a trait of interest, or the like, *etc.*, and is not limited to selectable, screenable or non-selectable marker genes. The inventors also contemplate that, where both an expressible gene that is not necessarily a marker gene is employed in combination with a marker gene, one may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

The choice of the particular DNA segments to be delivered to recipient cells with the constructs of the invention often will depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add commercially desirable, agronomically important traits to the plant. Such traits include, but are not limited to, herbicide resistance or tolerance; insect resistance or tolerance; disease resistance or tolerance (viral, bacterial, fungal, nematode); stress tolerance and/or resistance, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress, or oxidative stress; increased yields; food content and makeup; physical appearance; male sterility; drydown; standability; prolificacy; starch quantity and quality; oil quantity and quality; protein quality and

quantity; amino acid composition; and the like. One may desire to incorporate on the constructs of the invention one or more genes conferring any such desirable trait or traits, such as, for example, a gene or genes encoding herbicide resistance.

5        In certain embodiments, the present invention contemplates the transformation of a recipient cell with more than one advantageous transgene. Two or more transgenes can be supplied in a single transformation event using either distinct transgene-encoding vectors, or using a single vector incorporating two or more gene coding sequences. For example, plasmids bearing the *bar* and *aroA* expression units in either convergent, 10 divergent, or colinear orientation, are considered to be particularly useful. Further preferred combinations are those of an insect resistance gene, such as a Bt gene, along with a protease inhibitor gene such as *pinII*, or the use of *bar* in combination with either of the above genes. Of course, any two or more transgenes of any description, such as 15 those conferring herbicide, insect, disease (viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch properties, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired.

#### 1.      Herbicide Resistance

20        The genes encoding phosphinothricin acetyltransferase (*bar* and *pat*), glyphosate tolerant EPSP synthase genes, the glyphosate degradative enzyme gene *gox* encoding glyphosate oxidoreductase, *deh* (encoding a dehalogenase enzyme that inactivates dalapon), herbicide resistant (e.g., sulfonylurea and imidazolinone) acetolactate synthase, and *bxn* genes (encoding a nitrilase enzyme that degrades 25 bromoxynil) are good examples of herbicide resistant genes for use in transformation. The *bar* and *pat* genes code for an enzyme, phosphinothricin acetyltransferase (PAT), which inactivates the herbicide phosphinothricin and prevents this compound from inhibiting glutamine synthetase enzymes. The enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP Synthase), is normally inhibited by the herbicide 30 N-(phosphonomethyl)glycine (glyphosate). However, genes are known that encode glyphosate-resistant EPSP synthase enzymes. These genes are particularly contemplated for use in monocot transformation. The *deh* gene encodes the enzyme

dalapon dehalogenase and confers resistance to the herbicide dalapon. The *bxn* gene codes for a specific nitrilase enzyme that converts bromoxynil to a non-herbicultural degradation product.

5

## 2. Insect Resistance

Potential insect resistance genes that can be introduced include *Bacillus thuringiensis* crystal toxin genes or Bt genes (Watrud *et al.*, 1985). Bt genes may provide resistance to lepidopteran or coleopteran pests such as European Corn Borer (ECB). Preferred Bt toxin genes for use in such embodiments include the *CryIA(b)* and 10 *CryIA(c)* genes. Endotoxin genes from other species of *B. thuringiensis* which affect insect growth or development also may be employed in this regard.

It is contemplated that preferred Bt genes for use in the transformation protocols disclosed herein will be those in which the coding sequence has been 15 modified to effect increased expression in plants, and more particularly, in maize. Means for preparing synthetic genes are well known in the art and are disclosed in, for example, U.S. Patent No. 5,500,365 and U.S. Patent Number No. 5,689,052, each of the disclosures of which are specifically incorporated herein by reference in their entirety. Examples of such modified Bt toxin genes include a synthetic Bt *CryIA(b)* 20 gene (Perlak *et al.*, 1991), and the synthetic *CryIA(c)* gene termed 1800b (PCT Application WO 95/06128). Some examples of other Bt toxin genes known to those of skill in the art are given in Table 2 below.

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Table 2: *Bacillus thuringiensis* δ-Endotoxin Genes\*

New Nomenclature	Old Nomenclature	GenBank Accession #
Cry1Aa	CryIA(a)	M11250
Cry1Ab	CryIA(b)	M13898
Cry1Ac	CryIA(c)	M11068
Cry1Ad	CryIA(d)	M73250
Cry1Ae	CryIA(e)	M65252
Cry1Ba	CryIB	X06711

Table 2 – Continued

Cry1Bb	ET5	L32020
Cry1Bc	PEG5	Z46442
Cry1Bd	CryE1	U70726
Cry1Ca	CryIC	X07518
Cry1Cb	CryIC(b)	M97880
Cry1Da	CryID	X54160
Cry1Db	PrtB	Z22511
Cry1Ea	CryIE	X53985
Cry1Eb	CryIE(b)	M73253
Cry1Fa	CryIF	M63897
Cry1Fb	PrtD	Z22512
Cry1Ga	PrtA	Z22510
Cry1Gb	CryH2	U70725
Cry1Ha	PrtC	Z22513
Cry1Hb		U35780
Cry1Ia	CryV	X62821
Cry1Ib	CryV	U07642
Cry1Ja	ET4	L32019
Cry1Jb	ET1	U31527
Cry1K		U28801
Cry2Aa	CryIIA	M31738
Cry2Ab	CryIIB	M23724
Cry2Ac	CryIIC	X57252
Cry3A	CryIIIA	M22472
Cry3Ba	CryIIIB	X17123
Cry3Bb	CryIIIB2	M89794
Cry3C	CryIIID	X59797
Cry4A	CryIVA	Y00423
Cry4B	CryIVB	X07423
Cry5Aa	CryVA(a)	L07025

Table 2 – Continued

Cry5Ab	CryVA(b)	L07026
Cry6A	CryVIA	L07022
Cry6B	CryVIB	L07024
Cry7Aa	CryIIIC	M64478
Cry7Ab	CryIIICb	U04367
Cry8A	CryIIIE	U04364
Cry8B	CryIIIG	U04365
Cry8C	CryIIIF	U04366
Cry9A	CryIG	X58120
Cry9B	CryIX	X75019
Cry9C	CryIH	Z37527
Cry10A	CryIVC	M12662
Cry11A	CryIVD	M31737
Cry11B	Jeg80	X86902
Cry12A	CryVB	L07027
Cry13A	CryVC	L07023
Cry14A	CryVD	U13955
Cry15A	34kDa	M76442
Cry16A	cbm71	X94146
Cry17A	cbm71	X99478
Cry18A	CryBP1	X99049
Cry19A	Jeg65	Y08920
Cyt1Aa	CytA	X03182
Cyt1Ab	CytM	X98793
Cyt2A	CytB	Z14147
Cyt2B	CytB	U52043

<sup>a</sup>Adapted from: [http://epunix.biols.susx.ac.uk/Home/Neil\\_Crickmore/Bt/index.html](http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html)

5        *pinII*, from tomato or potato is envisioned to be particularly useful. Even more advantageous is the use of a *pinII* gene in combination with a Bt toxin gene, the combined effect of which has been discovered by the present inventors to produce synergistic insecticidal activity. Other genes which encode inhibitors of the insects' digestive system, or those that encode enzymes or co-factors that facilitate the production of inhibitors, also may be useful. This group may be exemplified by oryzacystatin and amylase inhibitors such as those from wheat and barley.

10        Also, genes encoding lectins may confer additional or alternative insecticide properties. Lectins (originally termed phytohemagglutinins) are multivalent carbohydrate-binding proteins which have the ability to agglutinate red blood cells from a range of species. Lectins have been identified recently as insecticidal agents with activity against weevils, ECB and rootworm (Murdock *et al.*, 1990; Czapla & Lang, 1990). Lectin genes contemplated to be useful include, for example, barley and wheat 15 germ agglutinin (WGA) and rice lectins (Gatehouse *et al.*, 1984), with WGA being preferred.

20        Genes controlling the production of large or small polypeptides active against insects when introduced into the insect pests, such as, *e.g.*, lytic peptides, peptide hormones and toxins and venoms, form another aspect of the invention. For example, it is contemplated that the expression of juvenile hormone esterase, directed towards specific insect pests, also may result in insecticidal activity, or perhaps cause cessation of metamorphosis (Hammock *et al.*, 1990).

25        Transgenic maize expressing genes which encode enzymes that affect the integrity of the insect cuticle form yet another aspect of the invention. Such genes include those encoding, *e.g.*, chitinase, proteases, lipases and also genes for the production of nikkomycin, a compound that inhibits chitin synthesis, the introduction of any of which is contemplated to produce insect resistant maize plants. Genes that code for activities that affect insect molting, such as those affecting the production of ecdysteroid UDP-glucosyl transferase, also fall within the scope of the useful transgenes 30 of the present invention.

5                   Genes that code for enzymes that facilitate the production of compounds that reduce the nutritional quality of the host plant to insect pests also are encompassed by the present invention. It may be possible, for instance, to confer insecticidal activity on a plant by altering its sterol composition. Sterols are obtained by insects from their diet and are used for hormone synthesis and membrane stability. Therefore alterations in plant sterol composition by expression of novel genes, *e.g.*, those that directly promote the production of undesirable sterols or those that convert desirable sterols into undesirable forms, could have a negative effect on insect growth and/or development 10 and hence endow the plant with insecticidal activity. Lipoxygenases are naturally occurring plant enzymes that have been shown to exhibit anti-nutritional effects on insects and to reduce the nutritional quality of their diet. Therefore, further embodiments of the invention concern transgenic plants with enhanced lipoxygenase activity which may be resistant to insect feeding.

15

15                   *Tripsacum dactyloides* is a species of grass that is resistant to certain insects, including corn root worm. It is anticipated that genes encoding proteins that are toxic to insects or are involved in the biosynthesis of compounds toxic to insects will be isolated from *Tripsacum* and that these novel genes will be useful in conferring resistance to insects. It is known that the basis of insect resistance in *Tripsacum* is genetic, because said resistance has been transferred to *Zea mays* via sexual crosses (Branson and Guss, 20 1972).

25

Further genes encoding proteins characterized as having potential insecticidal activity also may be used as transgenes in accordance herewith. Such genes include, for example, the cowpea trypsin inhibitor (CpTI; Hilder *et al.*, 1987) which may be used as a rootworm deterrent; genes encoding avermectin (*Avermectin* and *Abamectin*., Campbell, W.C., Ed., 1989; Ikeda *et al.*, 1987) which may prove particularly useful as a corn rootworm deterrent; ribosome inactivating protein genes; and even genes that regulate plant structures. Transgenic maize including anti-insect antibody genes and genes that code for enzymes that can convert a non-toxic insecticide (pro-insecticide)

applied to the outside of the plant into an insecticide inside the plant also are contemplated.

### 3. Environment or Stress Resistance

5 Improvement of a plant's ability to tolerate various environmental stresses such as, but not limited to, drought, excess moisture, chilling, freezing, high temperature, salt, and oxidative stress, also can be effected through expression of novel genes. It is proposed that benefits may be realized in terms of increased resistance to freezing temperatures through the introduction of an "antifreeze" protein  
10 such as that of the Winter Flounder (Cutler *et al.*, 1989) or synthetic gene derivatives thereof. Improved chilling tolerance also may be conferred through increased expression of glycerol-3-phosphate acetyltransferase in chloroplasts (Wolter *et al.*, 1992). Resistance to oxidative stress (often exacerbated by conditions such as chilling temperatures in combination with high light intensities) can be conferred by  
15 expression of superoxide dismutase (Gupta *et al.*, 1993), and may be improved by glutathione reductase (Bowler *et al.*, 1992). Such strategies may allow for tolerance to freezing in newly emerged fields as well as extending later maturity higher yielding varieties to earlier relative maturity zones.

20 It is contemplated that the expression of novel genes that favorably effect plant water content, total water potential, osmotic potential, and turgor will enhance the ability of the plant to tolerate drought. As used herein, the terms "drought resistance" and "drought tolerance" are used to refer to a plants increased resistance or tolerance to stress induced by a reduction in water availability, as compared to normal  
25 circumstances, and the ability of the plant to function and survive in lower-water environments. In this aspect of the invention it is proposed, for example, that the expression of genes encoding for the biosynthesis of osmotically-active solutes, such as polyol compounds, may impart protection against drought. Within this class are genes encoding for mannitol-L-phosphate dehydrogenase (Lee and Saier, 1982) and trehalose-6-phosphate synthase (Kaasen *et al.*, 1992). Through the subsequent action  
30 of native phosphatases in the cell or by the introduction and coexpression of a specific phosphatase, these introduced genes will result in the accumulation of either mannitol

or trehalose, respectively, both of which have been well documented as protective compounds able to mitigate the effects of stress. Mannitol accumulation in transgenic tobacco has been verified and preliminary results indicate that plants expressing high levels of this metabolite are able to tolerate an applied osmotic stress (Tarczynski *et al.*, 1992, 1993).

Similarly, the efficacy of other metabolites in protecting either enzyme function (e.g., alanopine or propionic acid) or membrane integrity (e.g., alanopine) has been documented (Loomis *et al.*, 1989), and therefore expression of genes encoding for the biosynthesis of these compounds might confer drought resistance in a manner similar to or complimentary to mannitol. Other examples of naturally occurring metabolites that are osmotically active and/or provide some direct protective effect during drought and/or desiccation include fructose, erythritol (Coxson *et al.*, 1992), sorbitol, dulcitol (Karsten *et al.*, 1992), glucosylglycerol (Reed *et al.*, 1984; Erdmann *et al.*, 1992), sucrose, stachyose (Koster and Leopold, 1988; Blackman *et al.*, 1992), raffinose (Bernal-Lugo and Leopold, 1992), proline (Rensburg *et al.*, 1993), glycine betaine, ononitol and pinitol (Vernon and Bohnert, 1992). Continued canopy growth and increased reproductive fitness during times of stress will be augmented by introduction and expression of genes such as those controlling the osmotically active compounds discussed above and other such compounds. Currently preferred genes which promote the synthesis of an osmotically active polyol compound are genes which encode the enzymes mannitol-1-phosphate dehydrogenase, trehalose-6-phosphate synthase and myoinositol 0-methyltransferase.

It is contemplated that the expression of specific proteins also may increase drought tolerance. Three classes of Late Embryogenic Proteins have been assigned based on structural similarities (see Dure *et al.*, 1989). All three classes of LEAs have been demonstrated in maturing (*i.e.* desiccating) seeds. Within these 3 types of LEA proteins, the Type-II (dehydrin-type) have generally been implicated in drought and/or desiccation tolerance in vegetative plant parts (*i.e.* Mundy and Chua, 1988; Piatkowski *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992). Recently, expression of a Type-III LEA (HVA-1) in tobacco was found to influence plant height, maturity and

drought tolerance (Fitzpatrick, 1993). In rice, expression of the HVA-1 gene influenced tolerance to water deficit and salinity (Xu *et al.*, 1996). Expression of structural genes from all three LEA groups may therefore confer drought tolerance. Other types of proteins induced during water stress include thiol proteases, aldolases and transmembrane transporters (Guerrero *et al.*, 1990), which may confer various protective and/or repair-type functions during drought stress. It also is contemplated that genes that effect lipid biosynthesis and hence membrane composition might also be useful in conferring drought resistance on the plant.

Many of these genes for improving drought resistance have complementary modes of action. Thus, it is envisaged that combinations of these genes might have additive and/or synergistic effects in improving drought resistance in corn. Many of these genes also improve freezing tolerance (or resistance); the physical stresses incurred during freezing and drought are similar in nature and may be mitigated in similar fashion. Benefit may be conferred via constitutive expression of these genes, but the preferred means of expressing these novel genes may be through the use of a turgor-induced promoter (such as the promoters for the turgor-induced genes described in Guerrero *et al.*, 1990 and Shagan *et al.*, 1993 which are incorporated herein by reference). Spatial and temporal expression patterns of these genes may enable corn to better withstand stress.

It is proposed that expression of genes that are involved with specific morphological traits that allow for increased water extractions from drying soil would be of benefit. For example, introduction and expression of genes that alter root characteristics may enhance water uptake. It also is contemplated that expression of genes that enhance reproductive fitness during times of stress would be of significant value. For example, expression of genes that improve the synchrony of pollen shed and receptiveness of the female flower parts, *i.e.*, silks, would be of benefit. In addition it is proposed that expression of genes that minimize kernel abortion during times of stress would increase the amount of grain to be harvested and hence be of value.

Given the overall role of water in determining yield, it is contemplated that enabling corn to utilize water more efficiently, through the introduction and expression of novel genes, will improve overall performance even when soil water availability is not limiting. By introducing genes that improve the ability of corn to 5 maximize water usage across a full range of stresses relating to water availability, yield stability or consistency of yield performance may be realized.

#### 4. Disease Resistance

It is proposed that increased resistance to diseases may be realized through 10 introduction of genes into plants, for example, into monocotyledonous plants such as maize. It is possible to produce resistance to diseases caused by viruses, bacteria, fungi and nematodes. It also is contemplated that control of mycotoxin producing organisms may be realized through expression of introduced genes.

15. Resistance to viruses may be produced through expression of novel genes. For example, it has been demonstrated that expression of a viral coat protein in a transgenic plant can impart resistance to infection of the plant by that virus and perhaps other closely related viruses (Cuozzo *et al.*, 1988, Hemenway *et al.*, 1988, Abel *et al.*, 1986). It is contemplated that expression of antisense genes targeted at 20 essential viral functions may impart resistance to said virus. For example, an antisense gene targeted at the gene responsible for replication of viral nucleic acid may inhibit replication and lead to resistance to the virus. It is believed that interference with other viral functions through the use of antisense genes also may increase resistance to viruses. Further, it is proposed that it may be possible to 25 achieve resistance to viruses through other approaches, including, but not limited to the use of satellite viruses. Examples of viral and viral-like diseases, for which one could introduce resistance to in a transgenic plant in accordance with the instant invention, are listed below, in Table 3.

Table 3: Plant Virus and Virus-like Diseases

DISEASE	CAUSATIVE AGENT
American wheat striate (wheat striate mosaic)	American wheat striate mosaic virus mosaic (AWSMV)
Barley stripe mosaic	Barley stripe mosaic virus (BSMV)
Barley yellow dwarf	Barley yellow dwarf virus (BYDV)
Brome mosaic	Brome mosaic virus (BMV)
Cereal chlorotic mottle*	Cereal chlorotic mottle virus (CCMV)
Corn chlorotic vein banding (Brazilian maize mosaic) <sup>1</sup>	Corn chlorotic vein banding virus (CCVBV)
Corn lethal necrosis	Virus complex (Maize chlorotic mottle virus[MCMV] and Maize dwarf mosaic virus [MDMV] A or B or Wheat streak mosaic virus[WSMV])
Cucumber mosaic	Cucumber mosaic virus (CMV)
Cynodon chlorotic streak*, <sup>1</sup>	Cynodon chlorotic streak virus (CCSV)
Johnsongrass mosaic	Johnsongrass mosaic virus (JGMV)
Maize bushy stunt	Mycoplasma-like organism (MLO) associated
Maize chlorotic dwarf	Maize chlorotic dwarf virus (MCDV)
Maize chlorotic mottle	Maize chlorotic mottle virus (MCMV)
Maize dwarf mosaic	Maize dwarf mosaic virus (MDMV) strains A, D, E and F
Maize leaf fleck	Maize leaf fleck virus (MLFV)
Maize line*	Maize line virus (MLV)
Maize mosaic (corn leaf stripe, enanismo rayado)	Maize mosaic virus (MMV)
Maize mottle and chlorotic stunt <sup>1</sup>	Maize mottle and chlorotic stunt virus*
Maize pellucid ringspot*	Maize pellucid ringspot virus (MPRV)
Maize raya gruesa*, <sup>1</sup>	Maize raya gruesa virus (MRGV)
maize rayado fino* (fine striping disease)	Maize rayado fino virus (MRFV)
Maize red leaf and red stripe*	Mollicute?
Maize red stripe*	Maize red stripe virus (MRSV)
Maize ring mottle*	Maize ring mottle virus (MRMV)
Maize rio IV*	Maize rio cuarto virus (MRCV)
Maize rough dwarf* (nanismo ruvido)	Maize rough dwarf virus (MRDV) (= Cereal tillering disease virus*)
Maize sterile stunt*	Maize sterile stunt virus (strains of barley yellow striate virus)
Maize streak*	Maize streak virus (MSV)
Maize stripe (maize chlorotic stripe, maize hoja blanca)	Maize stripe virus
Maize stunting*, <sup>1</sup>	Maize stunting virus
Maize tassel abortion*	Maize tassel abortion virus (MTAV)
Maize vein enation*	Maize vein enation virus (MVEV)
Maize wallaby ear*	Maize wallaby ear virus (MWEV)

Table 3- Continued

Maize white leaf*	Maize white leaf virus
Maize white line mosaic	Maize white line mosaic virus (MWLMV)
Millet red leaf*	Millet red leaf virus (MRLV)
Northern cereal mosaic*	Northern cereal mosaic virus (NCMV)
Oat pseudorosette* (zakuklivanie)	Oat pseudorosette virus
Oat sterile dwarf*	Oat sterile dwarf virus (OSDV)
Rice black-streaked dwarf*	Rice black-streaked dwarf virus (RBSDV)
Rice stripe*	Rice stripe virus (RSV)
Sorghum mosaic	Sorghum mosaic virus (SrMV), formerly sugarcane mosaic virus (SCMV) strains H, I and M
Sugarcane Fiji disease*	Sugarcane Fiji disease virus (FDV)
Sugarcane mosaic	Sugarcane mosaic virus (SCMV) strains A, B, D, E, SC, BC, Sabi and MB (formerly MDMV-B)
Vein enation*, <sup>1</sup>	Virus ?
Wheat spot mosaic <sup>1</sup>	Wheat spot mosaic virus (WSMV)

\*Not known to occur naturally on corn in the United States. <sup>1</sup> Minor viral disease.

5 It is proposed that increased resistance to diseases caused by bacteria and fungi may be realized through introduction of novel genes. It is contemplated that genes encoding so-called "peptide antibiotics," pathogenesis related (PR) proteins, toxin resistance, and proteins affecting host-pathogen interactions such as morphological characteristics will be useful. Peptide antibiotics are polypeptide sequences which are inhibitory to growth of bacteria and other microorganisms. For example, the classes of peptides referred to as cecropins and magainins inhibit growth of many species of bacteria and fungi. It is proposed that expression of PR proteins in monocotyledonous plants such as maize may be useful in conferring resistance to bacterial disease. These genes are induced following pathogen attack on a host plant and have been divided 10 amongst the PR proteins are  $\beta$ -1, 3-glucanases, chitinases, and osmotin and other proteins that are believed to function in plant resistance to disease organisms. Other genes have been identified that have antifungal properties, e.g., UDA (stinging nettle lectin) and hevein (Broekaert *et al.*, 1989; Barkai-Golan *et al.*, 1978). It is known 15 that certain plant diseases are caused by the production of phytotoxins. It is proposed that resistance to these diseases would be achieved through expression of a novel gene 20

that encodes an enzyme capable of degrading or otherwise inactivating the phytotoxin. It also is contemplated that expression of novel genes that alter the interactions between the host plant and pathogen may be useful in reducing the ability of the disease organism to invade the tissues of the host plant, e.g., an increase in the 5 waxiness of the leaf cuticle or other morphological characteristics. Examples of bacterial and fungal diseases, including downy mildews, for which one could introduce resistance to in a transgenic plant in accordance with the instant invention, are listed below, in Tables 4, 5 and 6.

10

**Table 4: Plant Bacterial Diseases**

DISEASE	CAUSATIVE AGENT
Bacterial leaf blight and stalk rot	<i>Pseudomonas avenae</i> subsp. <i>avenae</i>
Bacterial leaf spot	<i>Xanthomonas campestris</i> pv. <i>holcicola</i>
Bacterial stalk rot	<i>Enterobacter dissolvens</i> = <i>Erwinia dissolvens</i>
Bacterial stalk and top rot	<i>Erwinia carotovora</i> subsp. <i>carotovora</i> , <i>Erwinia chrysanthemi</i> pv. <i>zeae</i>
Bacterial stripe	<i>Pseudomonas andropogonis</i>
Chocolate spot	<i>Pseudomonas syringae</i> pv. <i>coronafaciens</i>
Goss's bacterial wilt and blight (leaf freckles and wilt)	<i>Clavibacter michiganensis</i> subsp. <i>nebraskensis</i> = <i>Corynebacterium michiganense</i> pv. <i>nebraskense</i>
Holcus spot	<i>Pseudomonas syringae</i> pv. <i>syringae</i>
Purple leaf sheath	Hemiparasitic bacteria + (See under Fungi)
Seed rot-seedling blight	<i>Bacillus subtilis</i>
Stewart's disease (bacterial wilt)	<i>Pantoea stewartii</i> = <i>Erwinia stewartii</i>
Corn stunt (achapparramiento, maize stunt, Mesa Central or Rio Grande maize stunt)	<i>Spiroplasma kunkelii</i>

**Table 5: Plant Fungal Diseases**

DISEASE	PATHOGEN
Anthracnose leaf blight and anthracnose stalk rot	<i>Colletotrichum graminicola</i> (teleomorph: <i>Glomerella graminicola</i> Politis), <i>Glomerella tucumanensis</i> (anamorph: <i>Glomerella falcatum</i> Went)
Aspergillus ear and kernel rot	<i>Aspergillus flavus</i> Link:Fr.
Banded leaf and sheath spot*	<i>Rhizoctonia solani</i> Kühn = <i>Rhizoctonia microsclerotia</i> J. Matz (teleomorph: <i>Thanatephorus cucumeris</i> )

Table 5 – Continued

Black bundle disease	<i>Acremonium strictum</i> W. Gams = <i>Cephalosporium acremonium</i> Auct. non Corda
Black kernel rot*	<i>Lasiodiplodia theobromae</i> = <i>Botryodiplodia theobromae</i> <i>Marasmiellus</i> sp.
Borde blanco*	<i>Physoderma maydis</i>
Brown spot (black spot, stalk rot)	<i>Acremonium strictum</i> = <i>Cephalosporium acremonium</i>
Cephalosporium kernel rot	<i>Macrophomina phaseolina</i> <i>Thanatephorus cucumeris</i> = <i>Corticium sasakii</i>
Charcoal rot	<i>Curvularia clavata</i> , <i>C. eragrostidis</i> , = <i>C. maculans</i> (teleomorph: <i>Cochliobolus eragrostidis</i> ), <i>Curvularia inaequalis</i> , <i>C. intermedia</i> (teleomorph: <i>Cochliobolus intermedius</i> ), <i>Curvularia lunata</i> (teleomorph: <i>Cochliobolus lunatus</i> ), <i>Curvularia pallens</i> (teleomorph: <i>Cochliobolus pallens</i> ), <i>Curvularia senegalensis</i> , <i>C. tuberculata</i> (teleomorph: <i>Cochliobolus tuberculatus</i> )
Corticium ear rot*	<i>Didymella exitalis</i>
Curvularia leaf spot	<i>Diplodia frumenti</i> (teleomorph: <i>Botryosphaeria festucae</i> )
Didymella leaf spot*	<i>Diplodia maydis</i> = <i>Stenocarpella maydis</i>
Diplodia ear rot and stalk rot	<i>Stenocarpella macrospora</i> = <i>Diplodia macrospora</i>
Diplodia ear rot, stalk rot, seed rot and seedling blight	
Diplodia leaf spot or leaf streak	

\*Not known to occur naturally on corn in the United States.

5

Table 6: Plant Downy Mildews

DISEASE	CAUSATIVE AGENT
Brown stripe downy mildew*	<i>Sclerotinia rayssiae</i> var. <i>zeae</i>
Crazy top downy mildew	<i>Sclerotinia macrospora</i> = <i>Sclerospora macrospora</i>
Green ear downy mildew (graminicola downy mildew)	<i>Sclerospora graminicola</i>
Java downy mildew*	<i>Peronosclerospora maydis</i> = <i>Sclerospora maydis</i>
Philippine downy mildew*	<i>Peronosclerospora philippinensis</i> = <i>Sclerospora philippinensis</i>
Sorghum downy mildew	<i>Peronosclerospora sorghi</i> = <i>Sclerospora sorghi</i>

Table 6 – Continued

Spontaneum downy mildew*	<i>Peronosclerospora spontanea</i> = <i>Sclerospora spontanea</i>
Sugarcane downy mildew*	<i>Peronosclerospora sacchari</i> = <i>Sclerospora sacchari</i>
Dry ear rot (cob, kernel and stalk rot)	<i>Nigrospora oryzae</i> (teleomorph: <i>Khuskia oryzae</i> )
Ear rots, minor	<i>Alternaria alternata</i> = <i>A. tenuis</i> , <i>Aspergillus glaucus</i> , <i>A. niger</i> , <i>Aspergillus</i> spp., <i>Botrytis cinerea</i> (teleomorph: <i>Botryotinia fuckeliana</i> ), <i>Cunninghamella</i> sp., <i>Curvularia</i> <i>pallescens</i> , <i>Doratomyces stemonitis</i> = <i>Cephalotrichum stemonitis</i> , <i>Fusarium</i> <i>culmorum</i> , <i>Gonatobotrys simplex</i> , <i>Pithomyces maydicus</i> , <i>Rhizopus</i> <i>microsporus</i> Tiegh., <i>R. stolonifer</i> = <i>R.</i> <i>nigricans</i> , <i>Scopulariopsis brumptii</i> . <i>Claviceps gigantea</i> (anamorph: <i>Sphacelia</i> sp.)
Ergot* (horse's tooth, diente de caballo)	<i>Aureobasidium zeae</i> = <i>Kabatiella zeae</i> <i>Fusarium subglutinans</i> = <i>F.</i> <i>moniliforme</i> var. <i>subglutinans</i>
Eyespot	<i>Fusarium moniliforme</i> (teleomorph: <i>Gibberella fujikuroi</i> )
Fusarium ear and stalk rot	<i>Fusarium avenaceum</i> (teleomorph: <i>Gibberella avenacea</i> )
Fusarium kernel, root and stalk rot, seed rot and seedling blight	<i>Gibberella zeae</i> (anamorph: <i>Fusarium</i> <i>graminearum</i> )
Fusarium stalk rot, seedling root rot	<i>Botryosphaeria zeae</i> = <i>Physalospora</i> <i>zeae</i> (anamorph: <i>Macrophoma zeae</i> )
Gibberella ear and stalk rot	<i>Cercospora sorghi</i> = <i>C. sorghi</i> var. <i>maydis</i> , <i>C. zeae-maydis</i>
Gray ear rot	<i>Exserohilum pedicellatum</i> = <i>Helminthosporium pedicellatum</i>
Gray leaf spot (Cercospora leaf spot)	(teleomorph: <i>Setosphaeria pedicellata</i> )
Helminthosporium root rot	<i>Cladosporium cladosporioides</i> = <i>Hormodendrum cladosporioides</i> , <i>C.</i> <i>herbarum</i> (teleomorph: <i>Mycosphaerella</i> <i>tassiana</i> )
Hormodendrum ear rot (Cladosporium rot)	<i>Hyalothyridium maydis</i>
Hyalothyridium leaf spot*	<i>Cephalosporium maydis</i>
Late wilt*	

Table 6 – Continued

Leaf spots, minor	<i>Alternaria alternata</i> , <i>Ascochyta maydis</i> , <i>A. tritici</i> , <i>A. zeicola</i> , <i>Bipolaris victoriae</i> = <i>Helminthosporium victoriae</i> (teleomorph: <i>Cochliobolus victoriae</i> ), <i>C. sativus</i> (anamorph: <i>Bipolaris</i> <i>sorokiniana</i> = <i>H. sorokinianum</i> = <i>H.</i> <i>sativum</i> ), <i>Epicoccum nigrum</i> , <i>Exserohilum prolatum</i> = <i>Drechslera</i> <i>prolata</i> (teleomorph: <i>Setosphaeria</i> <i>prolata</i> ) <i>Graphium penicilliooides</i> , <i>Leptosphaeria maydis</i> , <i>Leptothyrium</i> <i>zeae</i> , <i>Ophiophaerella herpotricha</i> , (anamorph: <i>Scopelosporiella</i> sp.), <i>Paraphaeosphaeria michotii</i> , <i>Phoma</i> sp., <i>Septoria zeae</i> , <i>S. zeicola</i> , <i>S. zeina</i> <i>Setosphaeria turcica</i> (anamorph: <i>Exserohilum turcicum</i> = <i>Helminthosporium turcicum</i> ) <i>Cochliobolus carbonum</i> (anamorph: <i>Bipolaris zeicola</i> = <i>Helminthosporium</i> <i>carbonum</i> ) <i>Penicillium</i> spp., <i>P. chrysogenum</i> , <i>P.</i> <i>expansum</i> , <i>P. oxalicum</i> <i>Phaeocystostroma ambiguum</i> , = <i>Phaeocystosporella zeae</i> <i>Phaeosphaeria maydis</i> = <i>Sphaerulina</i> <i>maydis</i> <i>Botryosphaeria festucae</i> = <i>Physalospora zeicola</i> (anamorph: <i>Diplodia frumenti</i> ) Hemiparasitic bacteria and fungi <i>Phoma terrestris</i> = <i>Pyrenopeziza</i> <i>terrestris</i> <i>Pythium</i> spp., <i>P. arrhenomanes</i> , <i>P.</i> <i>graminicola</i> <i>Pythium aphanidermatum</i> = <i>P. butleri</i> L. <i>Epicoccum nigrum</i>
Northern corn leaf blight (white blast, crown stalk rot, stripe)	
Northern corn leaf spot, Helminthosporium ear rot (race 1)	
Penicillium ear rot (blue eye, blue mold)	
Phaeocystostroma stalk rot and root rot	
Phaeosphaeria leaf spot*	
Physalospora ear rot (Botryosphaeria ear rot)	
Purple leaf sheath	
Pyrenopeziza stalk rot and root rot	
Pythium root rot	
Pythium stalk rot	
Red kernel disease (ear mold, leaf and seed rot)	
Rhizoctonia ear rot (sclerotial rot)	
Rhizoctonia root rot and stalk rot	

Table 6 – Continued

Root rots, minor	<i>Alternaria alternata, Cercospora sorghi, Dictochaeta fertilis, Fusarium acuminatum</i> (teleomorph: <i>Gibberella acuminata</i> ), <i>F. equiseti</i> (teleomorph: <i>G. intricans</i> ), <i>F. oxysporum</i> , <i>F. pallidoroseum</i> , <i>F. poae</i> , <i>F. roseum</i> , <i>G. cyanogenia</i> , (anamorph: <i>F. sulphureum</i> ), <i>Microdochium bolleyi</i> , <i>Mucor</i> sp., <i>Periconia circinata</i> , <i>Phytophthora cactorum</i> , <i>P. drechsleri</i> , <i>P. nicotianae</i> var. <i>parasitica</i> , <i>Rhizopus arrhizus</i>
Rostratum leaf spot (Helminthosporium leaf disease, ear and stalk rot)	<i>Setosphaeria rostrata</i> , (anamorph: <i>Exserohilum rostratum</i> = <i>Helminthosporium rostratum</i> )
Rust, common corn	<i>Puccinia sorghi</i>
Rust, southern corn	<i>Puccinia polyspora</i>
Rust, tropical corn	<i>Physopella pallescens</i> , <i>P. zaeae</i> = <i>Angiopsora zaeae</i>
Sclerotium ear rot* (southern blight)	<i>Sclerotium rolfsii</i> Sacc. (teleomorph: <i>Athelia rolfsii</i> )
Seed rot-seedling blight	<i>Bipolaris sorokiniana</i> , <i>B. zeicola</i> = <i>Helminthosporium carbonum</i> , <i>Diplodia maydis</i> , <i>Exserohilum pedicillatum</i> , <i>Exserohilum turcicum</i> = <i>Helminthosporium turcicum</i> , <i>Fusarium avenaceum</i> , <i>F. culmorum</i> , <i>F. moniliforme</i> , <i>Gibberella zaeae</i> (anamorph: <i>F. graminearum</i> ), <i>Macrohomina phaseolina</i> , <i>Penicillium</i> spp., <i>Phomopsis</i> sp., <i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>R. zaeae</i> , <i>Sclerotium rolfsii</i> , <i>Spicaria</i> sp.
Selenophoma leaf spot*	<i>Selenophoma</i> sp.
Sheath rot	<i>Gaeumannomyces graminis</i>
Shuck rot	<i>Myrothecium gramineum</i>
Silage mold	<i>Monascus purpureus</i> , <i>M. ruber</i>
Smut, common	<i>Ustilago zaeae</i> = <i>U. maydis</i>
Smut, false	<i>Ustilaginoidea virens</i>
Smut, head	<i>Sphacelotheca reiliana</i> = <i>Sporisorium holci-sorghii</i>
Southern corn leaf blight and stalk rot	<i>Cochliobolus heterostrophus</i> (anamorph: <i>Bipolaris maydis</i> = <i>Helminthosporium maydis</i> )

Table 6 – Continued

Southern leaf spot	<i>Stenocarpella macrospora</i> = <i>Diplodia macrospora</i>
Stalk rots, minor	<i>Cercospora sorghi</i> , <i>Fusarium episphaeria</i> , <i>F. merismoides</i> , <i>F. oxysporum</i> Schlechtend, <i>F. poae</i> , <i>F. roseum</i> , <i>F. solani</i> (teleomorph: <i>Nectria haematococca</i> ), <i>F. tricinctum</i> , <i>Mariannaea elegans</i> , <i>Mucor</i> sp., <i>Rhopographus zaeae</i> , <i>Spicaria</i> sp.
Storage rots	<i>Aspergillus</i> spp., <i>Penicillium</i> spp. and other fungi
Tar spot*	<i>Phyllachora maydis</i>
Trichoderma ear rot and root rot	<i>Trichoderma viride</i> = <i>T. lignorum</i> teleomorph: <i>Hypocrea</i> sp.
White ear rot, root and stalk rot	<i>Stenocarpella maydis</i> = <i>Diplodia zeae</i>
Yellow leaf blight	<i>Ascochyta ischaemi</i> , <i>Phyllosticta maydis</i> (teleomorph: <i>Mycosphaerella zeae-maydis</i> )
Zonate leaf spot	<i>Gloeocercospora sorghi</i>

\*Not known to occur naturally on corn in the United States.

5 Plant parasitic nematodes are a cause of disease in many plants, including maize. It is proposed that it would be possible to make a plant resistant to these organisms through the expression of novel genes. It is anticipated that control of nematode infestations would be accomplished by altering the ability of the nematode to recognize or attach to a host plant and/or enabling the plant to produce nematicidal compounds, including but not limited to proteins. Examples of nematode-associated 10 plant diseases, for which one could introduce resistance to in a transgenic plant in accordance with the invention are given below, in Table 7.

Table 7: Parasitic Nematodes

DISEASE	PATHOGEN
Awl	<i>Dolichodorus</i> spp., <i>D. heterocephalus</i>
Bulb and stem (Europe)	<i>Ditylenchus dipsaci</i>
Burrowing	<i>Radopholus similis</i>
Cyst	<i>Heterodera avenae</i> , <i>H. zeae</i> , <i>Punctodera chalcoensis</i>
Dagger	<i>Xiphinema</i> spp., <i>X. americanum</i> , <i>X. mediterraneum</i>
False root-knot	<i>Nacobbus dorsalis</i>

Table 7 – Continued

Lance, Columbia	<i>Hoplolaimus columbus</i>
Lance	<i>Hoplolaimus</i> spp., <i>H. galeatus</i>
Lesion	<i>Pratylenchus</i> spp., <i>P. brachyurus</i> , <i>P. crenatus</i> , <i>P. hexincisus</i> , <i>P. neglectus</i> , <i>P. penetrans</i> , <i>P. scribneri</i> , <i>P. thornei</i> , <i>P. zae</i>
Needle	<i>Longidorus</i> spp., <i>L. breviannulatus</i>
Ring	<i>Criconemella</i> spp., <i>C. ornata</i>
Root-knot	<i>Meloidogyne</i> spp., <i>M. chitwoodi</i> , <i>M. incognita</i> , <i>M. javanica</i>
Spiral	<i>Helicotylenchus</i> spp.
Sting	<i>Belonolaimus</i> spp., <i>B. longicaudatus</i>
Stubby-root	<i>Paratrichodorus</i> spp., <i>P. christiei</i> , <i>P. minor</i> , <i>Quinisulcius acutus</i> , <i>Trichodorus</i> spp.
Stunt	<i>Tylenchorhynchus dubius</i>

## 5. Mycotoxin Reduction/Elimination

5 Production of mycotoxins, including aflatoxin and fumonisin, by fungi associated with monocotyledonous plants such as maize is a significant factor in rendering the grain not useful. These fungal organisms do not cause disease symptoms and/or interfere with the growth of the plant, but they produce chemicals (mycotoxins) that are toxic to animals. It is contemplated that inhibition of the growth  
10 of these fungi would reduce the synthesis of these toxic substances and therefore reduce grain losses due to mycotoxin contamination. It also is proposed that it may be possible to introduce novel genes into monocotyledonous plants such as maize that would inhibit synthesis of the mycotoxin without interfering with fungal growth. Further, it is contemplated that expression of a novel gene which encodes an enzyme  
15 capable of rendering the mycotoxin nontoxic would be useful in order to achieve reduced mycotoxin contamination of grain. The result of any of the above mechanisms would be a reduced presence of mycotoxins on grain.

## 6. Grain Composition or Quality

Genes may be introduced into plants, particularly commercially important cereals such as maize, to improve the grain for which the cereal is primarily grown. A wide range of novel transgenic plants produced in this manner may be envisioned 5 depending on the particular end use of the grain.

The largest use of maize grain is for feed or food. Introduction of genes that alter the composition of the grain may greatly enhance the feed or food value. The primary components of maize grain are starch, protein, and oil. Each of these primary 10 components of maize grain may be improved by altering its level or composition. Several examples may be mentioned for illustrative purposes, but in no way provide an exhaustive list of possibilities.

The protein of cereal grains including maize is suboptimal for feed and food 15 purposes especially when fed to pigs, poultry, and humans. The protein is deficient in several amino acids that are essential in the diet of these species, requiring the addition of supplements to the grain. Limiting essential amino acids may include lysine, methionine, tryptophan, threonine, valine, arginine, and histidine. Some amino acids become limiting only after corn is supplemented with other inputs for feed 20 formulations. For example, when corn is supplemented with soybean meal to meet lysine requirements methionine becomes limiting. The levels of these essential amino acids in seeds and grain may be elevated by mechanisms which include, but are not limited to, the introduction of genes to increase the biosynthesis of the amino acids, decrease the degradation of the amino acids, increase the storage of the amino acids in 25 proteins, or increase transport of the amino acids to the seeds or grain.

One mechanism for increasing the biosynthesis of the amino acids is to introduce genes that deregulate the amino acid biosynthetic pathways such that the plant can no longer adequately control the levels that are produced. This may be done 30 by deregulating or bypassing steps in the amino acid biosynthetic pathway which are normally regulated by levels of the amino acid end product of the pathway. Examples include the introduction of genes that encode deregulated versions of the enzymes

aspartokinase or dihydrodipicolinic acid (DHDP)-synthase for increasing lysine and threonine production, and anthranilate synthase for increasing tryptophan production. Reduction of the catabolism of the amino acids may be accomplished by introduction of DNA sequences that reduce or eliminate the expression of genes encoding enzymes that catalyze steps in the catabolic pathways such as the enzyme lysine-ketoglutarate reductase.

The protein composition of the grain may be altered to improve the balance of amino acids in a variety of ways including elevating expression of native proteins, decreasing expression of those with poor composition, changing the composition of native proteins, or introducing genes encoding entirely new proteins possessing superior composition. Examples may include the introduction of DNA that decreases the expression of members of the zein family of storage proteins. This DNA may encode ribozymes or antisense sequences directed to impairing expression of zein proteins or expression of regulators of zein expression such as the opaque-2 gene product. It also is proposed that the protein composition of the grain may be modified through the phenomenon of co-suppression, *i.e.*, inhibition of expression of an endogenous gene through the expression of an identical structural gene or gene fragment introduced through transformation (Goring *et al.*, 1991). Additionally, the introduced DNA may encode enzymes which degrade zeins. The decreases in zein expression that are achieved may be accompanied by increases in proteins with more desirable amino acid composition or increases in other major seed constituents such as starch. Alternatively, a chimeric gene may be introduced that comprises a coding sequence for a native protein of adequate amino acid composition such as for one of the globulin proteins or 10 kD zein of maize and a promoter or other regulatory sequence designed to elevate expression of said protein. The coding sequence of said gene may include additional or replacement codons for essential amino acids. Further, a coding sequence obtained from another species, or, a partially or completely synthetic sequence encoding a completely unique peptide sequence designed to enhance the amino acid composition of the seed may be employed.

The introduction of genes that alter the oil content of the grain may be of value. Increases in oil content may result in increases in metabolizable-energy-content and density of the seeds for use in feed and food. The introduced genes may encode enzymes that remove or reduce rate-limitations or regulated steps in fatty acid or lipid biosynthesis. Such genes may include, but are not limited to, those that encode acetyl-CoA carboxylase, ACP-acyltransferase,  $\beta$ -ketoacyl-ACP synthase, plus other well known fatty acid biosynthetic activities. Other possibilities are genes that encode proteins that do not possess enzymatic activity such as acyl carrier protein. Genes may be introduced that alter the balance of fatty acids present in the oil providing a more healthful or nutritive feedstuff. The introduced DNA also may encode sequences that block expression of enzymes involved in fatty acid biosynthesis, altering the proportions of fatty acids present in the grain such as described below.

Genes may be introduced that enhance the nutritive value of the starch component of the grain, for example by increasing the degree of branching, resulting in improved utilization of the starch in cows by delaying its metabolism.

Besides affecting the major constituents of the grain, genes may be introduced that affect a variety of other nutritive, processing, or other quality aspects of the grain as used for feed or food. For example, pigmentation of the grain may be increased or decreased. Enhancement and stability of yellow pigmentation is desirable in some animal feeds and may be achieved by introduction of genes that result in enhanced production of xanthophylls and carotenes by eliminating rate-limiting steps in their production. Such genes may encode altered forms of the enzymes phytoene synthase, phytoene desaturase, or lycopene synthase. Alternatively, unpigmented white corn is desirable for production of many food products and may be produced by the introduction of DNA which blocks or eliminates steps in pigment production pathways.

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Feed or food comprising primarily maize or other cereal grains possesses insufficient quantities of vitamins and must be supplemented to provide adequate

nutritive value. Introduction of genes that enhance vitamin biosynthesis in seeds may be envisioned including, for example, vitamins A, E, B<sub>12</sub>, choline, and the like. Maize grain also does not possess sufficient mineral content for optimal nutritive value. Genes that affect the accumulation or availability of compounds containing phosphorus, sulfur, calcium, manganese, zinc, and iron among others would be valuable. An example may be the introduction of a gene that reduced phytic acid production or encoded the enzyme phytase which enhances phytic acid breakdown. These genes would increase levels of available phosphate in the diet, reducing the need for supplementation with mineral phosphate.

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Numerous other examples of improvement of maize or other cereals for feed and food purposes might be described. The improvements may not even necessarily involve the grain, but may, for example, improve the value of the corn for silage. Introduction of DNA to accomplish this might include sequences that alter lignin 15 production such as those that result in the "brown midrib" phenotype associated with superior feed value for cattle.

20

In addition to direct improvements in feed or food value, genes also may be introduced which improve the processing of corn and improve the value of the products resulting from the processing. The primary method of processing corn is via wetmilling. Maize may be improved though the expression of novel genes that increase the efficiency and reduce the cost of processing such as by decreasing steeping time.

25 30

Improving the value of wetmilling products may include altering the quantity or quality of starch, oil, corn gluten meal, or the components of corn gluten feed. Elevation of starch may be achieved through the identification and elimination of rate limiting steps in starch biosynthesis or by decreasing levels of the other components of the grain resulting in proportional increases in starch. An example of the former may be the introduction of genes encoding ADP-glucose pyrophosphorylase enzymes with altered regulatory activity or which are expressed at higher level. Examples of

the latter may include selective inhibitors of, for example, protein or oil biosynthesis expressed during later stages of kernel development.

The properties of starch may be beneficially altered by changing the ratio of 5 amylose to amylopectin, the size of the starch molecules, or their branching pattern. Through these changes a broad range of properties may be modified which include, but are not limited to, changes in gelatinization temperature, heat of gelatinization, clarity of films and pastes, rheological properties, and the like. To accomplish these changes in properties, genes that encode granule-bound or soluble starch synthase 10 activity or branching enzyme activity may be introduced alone or combination. DNA such as antisense constructs also may be used to decrease levels of endogenous activity of these enzymes. The introduced genes or constructs may possess regulatory sequences that time their expression to specific intervals in starch biosynthesis and starch granule development. Furthermore, it may be worthwhile to introduce and 15 express genes that result in the *in vivo* derivatization, or other modification, of the glucose moieties of the starch molecule. The covalent attachment of any molecule may be envisioned, limited only by the existence of enzymes that catalyze the derivatizations and the accessibility of appropriate substrates in the starch granule. Examples of important derivations may include the addition of functional groups such 20 as amines, carboxyls, or phosphate groups which provide sites for subsequent *in vitro* derivatizations or affect starch properties through the introduction of ionic charges. Examples of other modifications may include direct changes of the glucose units such as loss of hydroxyl groups or their oxidation to aldehyde or carboxyl groups.

25 Oil is another product of wetmilling of corn, the value of which may be improved by introduction and expression of genes. The quantity of oil that can be extracted by wetmilling may be elevated by approaches as described for feed and food above. Oil properties also may be altered to improve its performance in the production and use of cooking oil, shortenings, lubricants or other oil-derived 30 products or improvement of its health attributes when used in the food-related applications. Novel fatty acids also may be synthesized which upon extraction can serve as starting materials for chemical syntheses. The changes in oil properties may

be achieved by altering the type, level, or lipid arrangement of the fatty acids present in the oil. This in turn may be accomplished by the addition of genes that encode enzymes that catalyze the synthesis of novel fatty acids and the lipids possessing them or by increasing levels of native fatty acids while possibly reducing levels of precursors. Alternatively, DNA sequences may be introduced which slow or block steps in fatty acid biosynthesis resulting in the increase in precursor fatty acid intermediates. Genes that might be added include desaturases, epoxidases, hydratases, dehydratases, and other enzymes that catalyze reactions involving fatty acid intermediates. Representative examples of catalytic steps that might be blocked include the desaturations from stearic to oleic acid and oleic to linolenic acid resulting in the respective accumulations of stearic and oleic acids. Another example is the blockage of elongation steps resulting in the accumulation of C<sub>8</sub> to C<sub>12</sub> saturated fatty acids.

Improvements in the other major corn wetmilling products, corn gluten meal and corn gluten feed, also may be achieved by the introduction of genes to obtain novel corn plants. Representative possibilities include but are not limited to those described above for improvement of food and feed value.

In addition, it may further be considered that the corn plant be used for the production or manufacturing of useful biological compounds that were either not produced at all, or not produced at the same level, in the corn plant previously. The novel corn plants producing these compounds are made possible by the introduction and expression of genes by corn transformation methods. The vast array of possibilities include but are not limited to any biological compound which is presently produced by any organism such as proteins, nucleic acids, primary and intermediary metabolites, carbohydrate polymers, *etc.* The compounds may be produced by the plant, extracted upon harvest and/or processing, and used for any presently recognized useful purpose such as pharmaceuticals, fragrances, and industrial enzymes to name a few.

Further possibilities to exemplify the range of grain traits or properties potentially encoded by introduced genes in transgenic plants include grain with less breakage susceptibility for export purposes or larger grit size when processed by dry milling through introduction of genes that enhance  $\gamma$ -zein synthesis, popcorn with improved popping quality and expansion volume through genes that increase pericarp thickness, corn with whiter grain for food uses though introduction of genes that effectively block expression of enzymes involved in pigment production pathways, and improved quality of alcoholic beverages or sweet corn through introduction of genes which affect flavor such as the shrunken gene (encoding sucrose synthase) for sweet corn.

#### 7. Plant Agronomic Characteristics

Two of the factors determining where corn or other crops can be grown are the average daily temperature during the growing season and the length of time between frosts. Within the areas where it is possible to grow corn, there are varying limitations on the maximal time it is allowed to grow to maturity and be harvested. The corn to be grown in a particular area is selected for its ability to mature and dry down to harvestable moisture content within the required period of time with maximum possible yield. Therefore, corn of varying maturities is developed for different growing locations. Apart from the need to dry down sufficiently to permit harvest, it is desirable to have maximal drying take place in the field to minimize the amount of energy required for additional drying post-harvest. Also, the more readily the grain can dry down, the more time there is available for growth and kernel fill. It is considered that genes that influence maturity and/or dry down can be identified and introduced into corn lines using transformation techniques to create new corn varieties adapted to different growing locations or the same growing location, but having improved yield to moisture ratio at harvest. Expression of genes that are involved in regulation of plant development may be especially useful, e.g., the liguleless and rough sheath genes that have been identified in corn.

30

It is contemplated that genes may be introduced into monocots that would improve standability and other plant growth characteristics. Expression of novel

genes which confer stronger stalks, improved root systems, or prevent or reduce ear dropage would be of great value to the farmer. It is proposed that introduction and expression of genes that increase the total amount of photoassimilate available by, for example, increasing light distribution and/or interception would be advantageous. In 5 addition, the expression of genes that increase the efficiency of photosynthesis and/or the leaf canopy would further increase gains in productivity. It is contemplated that expression of a phytochrome gene in corn may be advantageous. Expression of such a gene may reduce apical dominance, confer semidwarfism on a plant, and increase shade tolerance (U.S. Patent No. 5,268,526). Such approaches would allow for 10 increased plant populations in the field.

Delay of late season vegetative senescence would increase the flow of assimilate into the grain and thus increase yield. It is proposed that overexpression of genes within corn that are associated with "stay green" or the expression of any gene 15 that delays senescence would be advantageous. For example, a nonyellowing mutant has been identified in *Festuca pratensis* (Davies *et al.*, 1990). Expression of this gene as well as others may prevent premature breakdown of chlorophyll and thus maintain canopy function.

#### 20 8. Nutrient Utilization

The ability to utilize available nutrients may be a limiting factor in growth of crop plants such as maize. It is proposed that it would be possible to alter nutrient uptake, tolerate pH extremes, mobilization through the plant, storage pools, and availability for metabolic activities by the introduction of novel genes. These 25 modifications would allow a plant such as maize to more efficiently utilize available nutrients. It is contemplated that an increase in the activity of, for example, an enzyme that is normally present in the plant and involved in nutrient utilization would increase the availability of a nutrient. An example of such an enzyme would be phytase. It is further contemplated that enhanced nitrogen utilization by the corn plant 30 is desirable. Expression of a glutamate dehydrogenase gene in maize, *e.g.*, *E. coli* *gdhA* genes, may lead to increased fixation of nitrogen in organic compounds. Furthermore, expression of *gdhA* in corn may lead to enhanced resistance to the

herbicide glufosinate by incorporation of excess ammonia into glutamate, thereby detoxifying the ammonia. It also is contemplated that expression of a novel gene may make a nutrient source available that was previously not accessible, e.g., an enzyme that releases a component of nutrient value from a more complex molecule, perhaps a 5 macromolecule.

#### 9. Male Sterility

Male sterility is useful in the production of hybrid seed. It is proposed that 10 male sterility may be produced through expression of novel genes. For example, it has been shown that expression of genes that encode proteins that interfere with development of the male inflorescence and/or gametophyte result in male sterility. Chimeric ribonuclease genes that express in the anthers of transgenic tobacco and oilseed rape have been demonstrated to lead to male sterility (Mariani *et al.*, 1990).

15 A number of mutations were discovered in maize that confer cytoplasmic male sterility. One mutation in particular, referred to as T cytoplasm, also correlates with sensitivity to Southern corn leaf blight. A DNA sequence, designated TURF-13 (Levings, 1990), was identified that correlates with T cytoplasm. It is proposed that it would be possible through the introduction of TURF-13 via transformation, to 20 separate male sterility from disease sensitivity. As it is necessary to be able to restore male fertility for breeding purposes and for grain production, it is proposed that genes encoding restoration of male fertility also may be introduced.

#### 10. Negative Selectable Markers

25 Introduction of genes encoding traits that can be selected against may be useful for eliminating undesirable linked genes. It is contemplated that when two or more genes are introduced together by cotransformation that the genes will be linked together on the host chromosome. For example, a gene encoding a *Bt* gene that confers insect resistance on the plant may be introduced into a plant together with a 30 *bar* gene that is useful as a selectable marker and confers resistance to the herbicide Liberty® on the plant. However, it may not be desirable to have an insect resistant plant that also is resistant to the herbicide Liberty®. It is proposed that one could

also introduce an antisense *bar* gene that is expressed in those tissues where one does not want expression of the *bar* gene, e.g., in whole plant parts. Hence, although the *bar* gene is expressed and is useful as a selectable marker, it is not useful to confer herbicide resistance on the whole plant. The *bar* antisense gene is a negative 5 selectable marker.

It also is contemplated that negative selection is necessary in order to screen a population of transformants for rare homologous recombinants generated through gene targeting. For example, a homologous recombinant may be identified through 10 the inactivation of a gene that was previously expressed in that cell. The antisense gene to neomycin phosphotransferase II (NPT II) has been investigated as a negative selectable marker in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Xiang. and Guerra, 1993). In this example, both sense and antisense NPT II genes are introduced into a plant through transformation and the resultant plants are sensitive to 15 the antibiotic kanamycin. An introduced gene that integrates into the host cell chromosome at the site of the antisense NPT II gene, and inactivates the antisense gene, will make the plant resistant to kanamycin and other aminoglycoside antibiotics. Therefore, rare, site-specific recombinants may be identified by screening for 20 antibiotic resistance. Similarly, any gene, native to the plant or introduced through transformation, that when inactivated confers resistance to a compound, may be useful as a negative selectable marker.

It is contemplated that negative selectable markers also may be useful in other ways. One application is to construct transgenic lines in which one could select for 25 transposition to unlinked sites. In the process of tagging it is most common for the transposable element to move to a genetically linked site on the same chromosome. A selectable marker for recovery of rare plants in which transposition has occurred to an unlinked locus would be useful. For example, the enzyme cytosine deaminase may be useful for this purpose (Stougaard, 1993). In the presence of this enzyme the 30 compound 5-fluorocytosine is converted to 5-fluorouracil which is toxic to plant and animal cells. If a transposable element is linked to the gene for the enzyme cytosine deaminase, one may select for transposition to unlinked sites by selecting for

transposition events in which the resultant plant is now resistant to 5-fluorocytosine. The parental plants and plants containing transpositions to linked sites will remain sensitive to 5-fluorocytosine. Resistance to 5-fluorocytosine is due to loss of the cytosine deaminase gene through genetic segregation of the transposable element and the cytosine deaminase gene. Other genes that encode proteins that render the plant sensitive to a certain compound will also be useful in this context. For example, T-DNA gene 2 from *Agrobacterium tumefaciens* encodes a protein that catalyzes the conversion of  $\alpha$ -naphthalene acetamide (NAM) to  $\alpha$ -naphthalene acetic acid (NAA) renders plant cells sensitive to high concentrations of NAM (Depicker *et al.*, 1988).

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It also is contemplated that negative selectable markers may be useful in the construction of transposon tagging lines. For example, by marking an autonomous transposable element such as Ac, Master Mu, or En/Spn with a negative selectable marker, one could select for transformants in which the autonomous element is not stably integrated into the genome. It is proposed that this would be desirable, for example, when transient expression of the autonomous element is desired to activate in trans the transposition of a defective transposable element, such as Ds, but stable integration of the autonomous element is not desired. The presence of the autonomous element may not be desired in order to stabilize the defective element, *i.e.*, prevent it from further transposing. However, it is proposed that if stable integration of an autonomous transposable element is desired in a plant the presence of a negative selectable marker may make it possible to eliminate the autonomous element during the breeding process.

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(vi) *Non-Protein-Expressing Sequences*

## 1. RNA-Expressing

DNA may be introduced into crop plants for the purpose of expressing RNA transcripts that function to affect plant phenotype yet are not translated into protein. Two examples are antisense RNA and RNA with ribozyme activity. Both may serve possible functions in reducing or eliminating expression of native or introduced plant genes.

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described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

Filters are washed in 2X SCP, 1% SDS at 65 C for 30 min. and visualized by autoradiography using Kodak XAR5 film. For rehybridization, the filters are boiled for 10 min. in distilled H<sub>2</sub>O to remove the first probe and then prehybridized as described above.

5

### EXAMPLE 13

#### Utilization of Transgenic Crops

The ultimate goal in plant transformation is to produce plants which are useful to man. In this respect, transgenic plants created in accordance with the current 10 invention may be used for virtually any purpose deemed of value to the grower or to the consumer. For example, one may wish to harvest seed from transgenic plants. This seed may in turn be used for a wide variety of purposes. The seed may be sold to farmers for planting in the field or may be directly used as food, either for animals or humans. Alternatively, products may be made from the seed itself. Examples of 15 products which may be made from the seed include, oil, starch, animal or human food, pharmaceuticals, and various industrial products. Such products may be made from particular plant parts or from the entire plant. One product made from the entire plant, which is deemed of particular value, is silage for animal feed.

20 Means for preparing products from plants, such as those that may be made with the current invention, have been well known since the dawn of agriculture and will be known to those of skill in the art. Specific methods for crop utilization may be found in, for example, Sprague and Dudley (1988), and Watson and Ramstad (1987).

25 All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of 30 steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents

## EXAMPLE 12

## General Methods for Assays

DNA analysis of transformed plants is performed as follows. Genomic DNA is isolated using a procedure modified from Shure, *et al.*, 1983. Approximately 1 gm 5 callus or leaf tissue is ground to a fine powder in liquid nitrogen using a mortar and pestle. Powdered tissue is mixed thoroughly with 4 ml extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate is extracted with 4 ml phenol/ chloroform. The aqueous phase is separated by centrifugation, passed through Miracloth, and precipitated twice using 10 1/10 volume of 4.4 M ammonium acetate, pH 5.2 and an equal volume of isopropanol. The precipitate is washed with 70% ethanol and resuspended in 200-500  $\mu$ l TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0).

15 The presence of a DNA sequence in a transformed cell may be detected through the use of polymerase chain reaction (PCR). Using this technique specific fragments of DNA can be amplified and detected following agarose gel electrophoresis. For example, two hundred to 1000 ng genomic DNA is added to a reaction mix containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1 mg/ml gelatin, 200 $\mu$ M each dATP, dCTP, dGTP, dTTP, 0.5 $\mu$ M each forward and 20 reverse DNA primers, 20% glycerol, and 2.5 units Taq DNA polymerase. The reaction is run in a thermal cycling machine as follows: 3 minutes at 94 C, 39 repeats of the cycle 1 minute at 94 C, 1 minute at 50 C, 30 seconds at 72 C, followed by 5 minutes at 72 C. Twenty  $\mu$ l of each reaction mix is run on a 3.5% NuSieve gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 50V for two to four hours.

25

For Southern blot analysis, genomic DNA is digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran (Schleicher and Schuell) using 10X SCP (20X SCP: 2 M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA). Filters are prehybridized 30 at 65°C in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500  $\mu$ g/ml heparin (Chomet *et al.*, 1987) for 15 min. Filters then are hybridized overnight at 65 C in 6X SCP containing 100  $\mu$  g/ml denatured salmon sperm DNA and <sup>32</sup>P-labeled probe.

saving significant time and effort. The gene controlling a trait being advanced in the breeding protocol may be genetically linked to the screenable marker as a result of being introduced into the host genome on a single segment of DNA which includes the screenable marker or selectable-screenable marker gene fusion, thereby forming a single transformation event in the host genome, or the gene or trait could be native to a chromosomal region which is in close proximity to the site of integration of the transgenic marker gene or marker gene fusion. The marker gene could also be introduced by co-transformation or multiple transformations, thereby leading to the production of multiple transformation events located in close genomic proximity.

10

A backcrossing protocol such as that described above typically proceeds five or more generations with selection for the desired trait at each generation, after which the progeny are hemizygous for loci controlling the characteristic being transferred, but are like the superior parent for most or almost all other genes. The last backcross generation are typically selfed to give progeny which are pure breeding for the gene(s) being transferred, *i.e.* one or more transformation events. With the instant invention, however, the last step is not required, as seeds could be selected from a set of heterozygous parents by identification of the screenable marker phenotype.

20

Therefore, through a series a breeding manipulations, a selected transgene may moved from one line into an entirely different line without the need for further recombinant manipulation. Transgenes are valuable in that they typically behave genetically as any other gene and can be manipulated by breeding techniques in a manner identical to any other corn gene. Therefore, one may produce inbreds plants which are true breeding for one or more transgenes. By crossing different inbred plants, one may produce a large number of different hybrids with different combinations of transgenes. In this way, plants may be produced which have the desirable agronomic properties frequently associated with hybrids ("hybrid vigor"), as well as the desirable characteristics imparted by one or more transgene(s).

25

30

In the case of selectable markers, selection may be carried out in solid or liquid media. The DNA constructs of the invention will preferably also include one or more genes which confer, either individually or in combination with other sequences, a desired phenotype on the transformed plant. Exemplary genes for transformation and the corresponding phenotypes these sequences may confer on the transformed plant are disclosed herein.

### EXAMPLE 11

#### Plant Breeding Protocols Using Seed-Expressed Screenable Markers

10 The constructs of the instant invention will be especially useful in plant breeding programs. In particular, the invention facilitates the advancement of transgene constructs into subsequent generations by allowing direct screening of transgenic seeds at each generation. An example of a breeding protocol for which the invention can be applied is backcrossing. Backcrossing may be used to transfer a 15 selected trait to a plant originally lacking the trait. In the case of the current invention, the selected trait is comprised on a transgene which includes a screenable marker, or is genetically linked thereto. The advantage of the instant invention is that the presence of this transgene can be ascertained directly in viable seeds without the need for destructive assays or growing of the seed. In particular, transgenic seeds can be 20 identified based on the expression of a screenable marker.

Therefore, plants comprising the constructs of the current invention may be entered into breeding protocols which utilize the transgenic seed screening capabilities provided by these constructs. For example, in a conventional breeding protocol, a 25 superior inbred (A) (recurrent parent) is crossed to a donor inbred (non-recurrent parent), which carries the appropriate gene(s) for the trait in question. The progeny of this cross are then selected which comprise the desired trait to be transferred from the non-recurrent parent, then the selected progeny are mated back to the superior recurrent parent (A). This process requires the time and labor intensive steps of 30 growing of the progeny seeds and assaying the resultant plants for the desired trait at each generation. However, the instant invention allows the step of selecting a trait which is genetically linked to a screenable marker based on the phenotype of the seed,

nicotinic acid, 3% sucrose, 3.6 g/L Gelgro, pH 5.8) containing 100 mg/L paromomycin in Phytatrays (Sigma) and cultured at about 27°C in the light. Plantlets that develop in Phytatrays after 3-6 weeks are then transferred to soil. Plantlets are acclimated in a growth chamber and grown to maturity in the greenhouse.

5

## EXAMPLE 10

### General Methods for Microprojectile Bombardment

Many variations in techniques for microprojectile bombardment are well known in the art and therefore deemed useful with the current invention. Exemplary 10 procedures for bombardment are discussed in, for example, U.S. Patent Serial No. 08/113,561, filed August 25, 1993. Examples of target tissues which may be used with the current invention include immature embryos, Type I callus, Type II callus, Type III callus, suspension cultures and meristematic tissue (PCT Publication WO 96/04392). Some genotypes which are especially useful for maize transformation are 15 disclosed in, for example, U.S. Patent Serial No. 08/113,561, filed August 25, 1993. Preferred genotypes will be those which are readily transformable and which also may be regenerated to yield a fertile transgenic plant.

Any method for acceleration of microprojectiles may potentially be used to 20 transform a plant cell with the current invention. A preferred method will be a gas-driven particle gun such as the DuPont Biolistics PDS1000He particle bombardment device. Exemplary particles for bombardment include those comprised of tungsten, gold, platinum, and the like. Gold particles are deemed particularly useful in the current invention, with 0.6  $\mu\text{m}$  or 0.7  $\mu\text{m}$  gold particles being preferred and 0.6  $\mu\text{m}$  25 particles most preferred. The most preferred particles will be DNA coated and have a mean size between 0.6  $\mu\text{m}$  and 1.0  $\mu\text{m}$ .

As disclosed herein, any DNA sequence may potentially be used for 30 transformation. The DNA segments used for transformation will include a selectable and/or screenable marker construct in accordance with the current invention. Many examples of selectable and screenable marker genes which may be used with constructs prepared in accordance with the invention are specifically disclosed herein.

For the luciferase analysis of seeds, seeds are immersed into a petri dish containing 150  $\mu$ l of 1 mM potassium luciferin (Analytical Luminescence Laboratories, La Jolla, CA) in 67 mM sodium Mes, pH 5.4. For observation, a starlight scope with relay lens (for example, a light amplifier NVS100; OpticElectronic, Dallas) and a highly sensitive video camera are used, *e.g.*, a photon counting camera (Hamanatsu Corp., Bridgewater, NJ)). Preferably observations are made about 20 min after administration of luciferin. The presence of the R-gene screenable marker can be scored by the color of the seed, with transformed seeds exhibiting a red color.

#### EXAMPLE 9

##### Transformation of H99 Immature Embryos or Callus and Selection with Paromomycin

Maize immature embryos (1.2 - 3.0 mm, 10-14 days post pollination) are isolated from greenhouse grown H99 plants that have been self or sib pollinated. Immature embryos are cultured on 735 medium in the dark at approximately 27°C. Immature embryos are either bombarded 1-6 days after isolation or cultured to produce embryogenic callus that is used for bombardment. Embryogenic callus is expanded and maintained by subculturing at 2-3 week intervals to fresh 735 medium. Prior to bombardment, cultured embryos or embryogenic callus (subdivided in approximately 2-4 mm clumps) are transferred to 735 medium containing 12% sucrose for 3-6 hours. Following bombardment, carried out as described in Example 7, tissue cultures are incubated overnight and transferred to 735 medium containing 500 mg/L paromomycin. After 2-3 weeks, callus is subdivided into small pieces (approximately 2-4 mm in diameter) and transferred to fresh selection medium. This subculture step is repeated at 2-3 week intervals for up to about 15 week post-bombardment, with subdivision and visual selection for healthy, growing callus.

Paromomycin tolerant callus is transferred to 735 medium without 2,4-D but containing 3.52 mg/L BAP for 3-9 days in the dark at approximately 27°C and is subsequently transferred to 110 medium (1/2X MS salts, 0.5 mg/L thiamine, 0.5 mg/L

Two hundred twenty microliters sterile water, 250  $\mu$ l 2.5 M CaCl<sub>2</sub> and 50  $\mu$ l stock spermidine (14  $\mu$ l spermidine in 986  $\mu$ l water) were then added to the particle containing solution. The solution was thoroughly mixed and placed on ice, followed by vortexing at 4°C for 10 minutes and centrifugation at 500 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in 600  $\mu$ l absolute ethanol. Following centrifugation at 500 rpm for 5 minutes, the pellet was resuspended in 36-38  $\mu$ l of absolute ethanol, vortexed for approximately 20 seconds, and sonicated for 20-30 seconds. At this stage the particles were allowed to sit for 2-5 minutes, after which 5-10  $\mu$ l of the supernatant was removed and dispensed on the surface of a flyer disk and the ethanol allowed to dry completely. Alternatively, particles may be removed directly after resuspension and vortexing 20 to 30 seconds in 36  $\mu$ l-38  $\mu$ l of ethanol, placed on the flyer disk and allowed to dry as done for the settled treatment.

The particles were then used for bombardment of the embryos or SC716 suspension cultures by a helium blast of approximately 1100 psi using the DuPont Biolistics PDS1000He particle bombardment device. The plates containing embryos were placed on the third shelf from the bottom, 5 cm below the stopping screen. Each plate was bombarded once with the DuPont Biolistics PDS1000He particle gun. Cells and embryos were bombarded and incubated in the dark at approximately 27°C. GFP was visualized about 48 hours post bombardment.

#### EXAMPLE 8

##### Screenable Marker Assay and Photography

For GFP analysis, seeds were exposed to ultraviolet light and visually scored. Alternatively, if fluorescence is faint, scoring may be enhanced utilization of devices that enhance night vision, or by a highly light sensitive video camera, such as a photon counting camera. Detection will be enhanced by the utilization of specialized filters for excitation and detection of fluorescence. In the instant case, transgenic tissue was observed with a Zeiss Axiophot microscope. Fluorescence was supplied by a mercury light source and appropriate filter set (an exciter filter (BP450-490), a chromatic beam splitter (FT510), and a barrier filter (BP515-565) (Carl Zeiss, Inc., Thornwood, NY).

## EXAMPLE 7

**Microprojectile Bombardment Transformation of Immature Embryos and Suspension Cultures**

SC716 suspension culture cells were plated onto Whatman #4 paper filters (7 cm) using a Bucher funnel. Filters with suspension culture cells (approximately 0.5 ml PCU/filter) were transferred to 735 medium containing 12% sucrose prior to bombardment. Alternatively, immature embryos (1.2 - 3.0 mm in length) were excised from surface-sterilized, greenhouse-grown ears of Hi-II 10-12 days post-pollination. The Hi-II genotype was developed from an A188 x B73 cross (Armstrong *et al.*, 1991). Approximately 30 embryos per petri dish were plated axis side down on a modified N6 medium containing 1 mg/l 2,4-D, 100 mg/l casein hydrolysate, 6 mM L-proline, 0.5 g/l 2-(N-morpholino) ethanesulfonic acid (MES), 0.75 g/l MgCl<sub>2</sub>, and 2% sucrose solidified with 2 g/l Gelgro, pH 5.8 (#735 medium). Embryos were cultured in the dark for two to four days at 24°C.

15

Approximately 3-4 hours prior to bombardment, embryos were transferred to the above culture medium with the sucrose concentration increased from 3% to 12%. When embryos were transferred to the high osmoticum medium they were arranged in concentric circles on the plate, starting 1 cm from the center of the dish, positioned such that their coleorhizal end was oriented toward the center of the dish. Usually two concentric circles were formed with 25-35 embryos per plate.

25

Microprojectiles were prepared for transformation by adding 60 mg of 0.6 µm gold microprojectiles (BioRad, cat. no. 165-2262) to 1000 µl absolute ethanol and incubating for at least 3 hours at room temperature followed by storage at -20°C. Twenty to thirty five µl of the sterile gold particles and more preferably 30 to 35 µl of gold particles (30 µl contains 1.8 mg of particles) was then centrifuged in a microcentrifuge for up to 1 min. The supernatant was removed and one ml sterile water was added to the tube, followed by centrifugation at 1800-2000 rpm for 2-5 minutes. Microprojectile particles were resuspended in 25-30 µl of DNA solution containing approximately 1-1000 µg per each 1.8 mg of particles.

swirling the original flask to suspend the cells (which tend to settle to the bottom of the culture vessel), tilting the flask on its side and allowing the denser cells and cell aggregates to settle slightly. One ml of packed cells was then drawn off from this pool of settled cells together with 4 ml of conditioned medium and added to a flask containing 20 ml fresh medium. A sterile ten ml, wide tip, pipet was used for this transfer (Falcon 7304). Any very large aggregates of cells which would not pass easily through the pipet tip were excluded. If a growth regulator capsule was present, it was also transferred to the new flask.

After approximately 7 weeks, the loose embryogenic cell aggregates began to predominate and fragment in each of the cultures, reached a state referred to as "dispersed." The treatment which yielded the highest proportion of embryogenic clusters was the 402 medium plus one NAA capsule. After the cultures became dispersed and were doubling approximately every two to three days as determined by increase in packed cell volume, a one ml packed cell volume inoculum from each culture was transferred into 20 ml 401 medium using a ten ml narrow tip pipet (Falcon 7551). These transfers were performed about every 3½ days. An inoculum from the 402 plus 2,4-D plus NAA capsules culture was also used to initiate a culture in 409 medium (402 without 2,4-D and including 10mg/l dicamba) either with or without 1 ml coconut water (Gibco 670-8130AG) per 25 ml culture medium. The most dispersed cultures were cryopreserved after 2 weeks, 2 months or 5 months.

The culture grown on 409 with coconut water was thawed approximately 3.5 years after cryopreservation, cultured for two weeks on solid 201 (except containing 0.5 mg/kg 2,4-D, 2% manitol, and 3% sucrose) culture medium using BMS as a feeder layer (Rhodes *et al.*, 1988) and transferred to media 409 without coconut water. The culture was maintained by subculturing once or twice weekly in 409 or 401 medium by the method described above.

**Table 11: Visual Estimate of GFP Expression in (1X6)716 Suspension Cells and (AWXCW) Immature Embryos at 48 H Post-Bombardment**

GFP Vector	Expression in 716	Expression in AW X
	Suspension	<i>HiII</i> Immature Embryos
35S-GFP	+++	+++
L3/MGFP::NPTII/Tr7	++	++++
L3/EGFP::NPTII/Tr7	++	++++
L3/rACT/MGFP::NPTII/Tr7	++++	++++
L3/rACT/EGFP::NPTII/Tr7	++++	++++

#### EXAMPLE 6

5                   **Initiation of the Suspension Culture G(A188XB73)716 (designated 716)  
for Use in Transformation**

The maize suspension culture designated 716, which was employed by the instant inventors for transformation studies with selectable-screenable marker gene fusions, was prepared as follows. Type II tissue used to initiate the cell suspension 10 was initiated from immature embryos of A188 x B73 plated onto N6-based medium with 1 mg/ml 2,4-D (201; see Table 8). A Type II callus was initiated by visual selection of fast growing, friable embryogenic cells. The suspension was initiated within 6 months after callus initiation. Tissue chosen from the callus to initiate the 15 suspension consisted of undifferentiated Type II callus. The characteristics of this undifferentiated tissue include the earliest stages of embryo development and soft, friable, undifferentiated tissue underlying it.

Approximately one gram of tissue was added to 20 ml of liquid medium. In 20 this example, the liquid medium was medium 402 to which different slow-release growth regulator capsule treatments were added (U.S. Patent No. 5,550,318). These capsule treatments included 2,4-D, NAA, 2,4-D plus NAA, and two NAA capsules. One flask was initiated for each of the different 402 media plus growth regulator combinations. Every 7 days each culture was subcultured into fresh medium by transferring a small portion of the cellular suspension to a new flask. This involved

cells. Also, the Actin intron appeared to boost expression in the suspension cells, whereas expression in the embryos was high regardless of the presence of the intron.

**Table 9: Vectors Tested by Transient Assay**

Plasmid number	GFP Expression Cassette
pDPG720	35S-GFP-TYG-nos
pDV130	L3/MGFP::NPTII/Tr7
pDV131	L3/EGFP::NPTII/Tr7
pDV132	L3/rACT/MGFP::NPTII/Tr7
pDV133	L3/rACT/EGFP::NPTII/Tr7

5

**Table 10: Components of GFP Fusion gene Constructs.**

Gene Element	Description	Reference	GenBank Accession
L3 promoter	promoter from maize ole 16 gene	Lee and Huang, <i>Plant Mol. Biol.</i> , 26:1981-1987, 1994	U13701
MGFP	enhanced GFP sequence	Pang <i>et al.</i> , <i>Plant Physiology</i> , 112:893-900, 1996	
EGFP	enhanced GFP sequence (Clontech cat#6077-1)	Yang <i>et al.</i> , <i>Nucleic Acids Res.</i> , 24:4592-4593, 1996	U76561
GFP-TYG	enhanced GFP sequence	Chiu <i>et al.</i> , <i>Curr. Biol.</i> , 1996 6:325-330, 1996	
rACT	rice actin1 intron1	McElroy <i>et al.</i> , <i>Plant Cell</i> , 2:163-171, 1990	
Tr7	<i>Agrobacterium tumefaciens</i> <td>Dhaese <i>et al.</i>, <i>EMBO J.</i>, 2:419-426, 1983</td> <td></td>	Dhaese <i>et al.</i> , <i>EMBO J.</i> , 2:419-426, 1983	

manufacturer, with the addition of IPTG to 1 mM in the liquid LB growth medium. Cells were plated out on LB-agar (Gibco Life Sciences) containing 0.0008% X-gal and 0.2 mM IPTG and supplemented with either kanamycin (50 µg/ml) or ampicillin (100 µg/ml). Colonies were observed on both kanamycin and ampicillin media from 5 the transformation involving pBSK/EGFP::NPTII, indicating the presence of NPTII activity as determined by resistance of the colonies to kanamycin. Colonies resulting from transformation with pBSK/MGFP::NPTII were observed only on ampicillin media, and not on kanamycin media. The growth of colonies on ampicillin media in 10 both transformation experiments was the result of expression of the beta-lactamase gene resident in pBSK; the absence of colonies on kanamycin media in the pBSK/MGFP::NPTII transformation experiment was likely due to the fact that the MGFP sequence contains the potato ST-LS1 intron which is not removed from the 15 NGFP::NPTII transcript in the *E. coli* host. Analysis of pBSK/EGFP::NPTII colonies using fluorescent microscopy allowed for detection of fluorescence of GFP, indicating the presence of both *nptII* and GFP activity in these cells.

#### EXAMPLE 5

##### Transformation of Hi x AW Immature Embryos and 716 Suspensions With GFP Fusion Gene Constructs

20 Immature embryos (Hi X AW) and 716 suspension were used to study transient expression of vectors comprising selectable-screenable marker gene fusions. The makeup of the vectors used is listed below, in Table 9, and given in FIGs. 1-4, and the components included in the vectors are given in Table 10. Two plates of each tissue were used for each of the vectors. A 35S-GFP vector was used as a control 25 (pDPG720). Approximately 0.5 µg of plasmid DNA was used for each plate (2.0 µg/tube). Microprojectile bombardment was carried out as described in Example 7. Bombarded tissue was observed with a Zeiss Axiophot microscope. Fluorescence was supplied by a mercury light source and a filter set comprising a BP450-490 exciter filter, a FT510 chromatic beam splitter, and a BP515-565 barrier filter (Carl 30 Zeiss, Inc., Thornwood, NY). GFP expression was observed for each of the vectors. Table 11 shows a visual estimate of GFP expression 48 h post-bombardment. It was noted that the L3 promoter seemed to express better in the embryos than in suspension

Bellafonte, PA). This product was then cleaved with EcoRI and ligated into the EcoRI site of pDPG778 by using the Rapid Ligation Kit (Boehringer Mannheim, Indianapolis, IN). A clone was selected by cleaving with BamHI and KpnI, which provides a diagnostic of fragments of 16, 333, 563, 717, 1921 and 3391 bp. This 5 clone was designated pDPG779.

#### EXAMPLE 4

##### Bacterial Expression of GFP:NPTII Constructs

The plasmid DV127 (SEQ ID NO:1), prepared as described in Example 1, was 10 cleaved with *EcoRV* and *HindIII* to release a fragment of 1736 bp containing the MGFP::NPTII sequence. The fragment was isolated by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA) according to the manufacturers directions. This fragment was inserted into the plasmid pBSK (Stratagene, La Jolla, CA) which had been cleaved 15 with *EcoRV* and *HindIII* using the components of the Rapid Ligation Kit (Boehringer Mannheim, Indianapolis, IN). This placed the MGFP::NPTII fusion protein in frame with the beta-galactosidase coding sequence resident in the pBSK plasmid under the control of the *E. coli* lac-i promoter. This plasmid was designated pBSK/MGFP::NPTII (FIG. 6).

20

Similarly, pDPG899 (DV134; SEQ ID NO:6) was cleaved with *NotI* and *HindIII* to release a 1621 bp fragment containing the EGFP::NPTII. This fragment was isolated by gel electrophoresis, band excision, and DNA elution using the Genelute kit (Supelco; Bellafonte, PA). This fragment was inserted into the plasmid 25 pBSK (Stratagene, La Jolla, CA), which had been cleaved with *NotI* and *HindIII* by using the components of the Rapid Ligation Kit (Boehringer Mannheim, Indianapolis, IN). This placed the EGFP::NPTII fusion protein in frame with the beta-galactosidase coding sequence resident in the pBSK plasmid under control of the *E. coli* lac-i promoter. This plasmid was designated pBSK/EGFP::NPTII (FIG. 5).

30

DH5 $\alpha$  cells (Gibco Life Sciences, Bethesda, MD) were transformed with either pBSK/MGFP::NPTII or pBSK/EGFP::NPTII, as recommended by the

For construction of the plasmid vector pDPG779, pDPG778 DNA was cleaved with EcoRI and treated with alkaline phosphatase to prevent self ligation. The rice ActinI IntronI sequence was amplified in a PCR in which pDM302 (Plant Cell Reports 11:586-591, 1992) was used as a template and oligonucleotides pRACT 5I (SEQ ID NO:18) and rACT 3I (SEQ ID NO:19) as primers; these primers also served to add EcoRI ends to the amplification product. Each primer containing an EcoRI tail for cloning.

10 rACT 5I - GGAATTCCGGTAACCACCCGCCCTCT  
rACT 3I - GGAATTCTACCTACAAAAAGCTCCG

PCR amplification of the rice ActinI IntronI sequence from pDM302 was performed as follows:

15 HF PCR Kit (Boehringer Mannheim, Indianapolis, IN)  
500 nM each primer  
200 uM dNTP's  
100 pg template (pDM302; Plant Cell Reports (1992) 11:586-591)  
20 3 mM MgCl

Cycling:

MJ Research PTC-100

95° - 2 min. 1 cycle  
25 94° - 45 sec.  
56° - 45 sec.  
72° - 45 sec. 32 cycles  
72° - 4 min. 1 cycle  
4° - hold

30

The expected 472 bp fragment was purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute system (Supelco,

when clone "L3 1050-4" was chosen from this ligation. The actual length of the final promoter sequence was 1039 bp. The L3 ClaIB sequence was:

L3 ClaIB                    TTGCTGAAGGTGAGATGC

5

**EXAMPLE 3**  
**Preparation of L3/GUS Constructs**

The preparation of the vector pDPG778 first involved construction of pDPG684, which contains a multiple cloning site immediately upstream of the GUS coding sequence. pDPG684 was constructed as follows: pDPG126 (35S/GUS/Tr7) was cleaved with EcoRI and BamHI in order to isolate a 2370 bp fragment containing the GUS coding sequence and Tr7 terminator. A fragment of the expected size was observed in agarose gel electrophoresis, and this was excised from the gel and purified using the Elu-Quik Kit (Schleicher and Schuell, Keene, NH). The cohesive ends of this fragment were then filled in using Klenow enzyme (DNA polymerase I, Boehringer Mannheim, Indianapolis, IN). The vector pBSK(-) (Stratagene, LaJolla, CA) was cleaved with SalI and the cohesive ends filled in the same manner followed by treatment with alkaline phosphatase to prevent self ligation. These two fragments were then ligated using the Rapid Ligation Kit (Boehringer Mannheim, Indianapolis, IN) and clones were selected by cleavage with EcoRV, which provides a diagnostic set of fragments of 231, 604, 1308 and 3257 bp. This clone was designated pDPG684.

The L3 promoter (1039 bp) was excised from clone "L3 1050-4," prepared as described in Example 2, by cleavage with XbaI and EcoRI, and the expected 1064 bp band was purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute system (Supelco, Bellafonte, PA). The plasmid pDPG684 (polylinker/GUS/Tr7) was also cleaved with XbaI and EcoRI and the 5370 bp backbone was purified by the same method. The 1039 bp L3 promoter fragment were inserted into the cleaved pDPG684 backbone in a ligation reaction using the Rapid Ligation Kit (Boehringer Mannheim, Indianapolis, IN). A single clone was selected and designated pDPG778.

band, which was purified by gel excision and EluQuick and subsequently cloned by T/A cloning into pBSK (Stratagene, La Jolla, CA) which had been cleaved with EcoRV and treated with terminal nucleotide transferase and dideoxy dTTP (Promega, Madison, WI) to create T overhangs. The PCR product was cloned using the Rapid 5 Ligation Kit (Boehringer Mannheim, Indianapolis, IN). A recombinant plasmid containing the insert was designated "t/aL3 1-3." By restriction digestion of t/aL3 1-3 with EcoRI and NotI (which cuts 165 bp upstream of the ATG initiating codon), a 200 bp fragment was removed and sub-cloned into pcDNAII (Invitrogen) cleaved with EcoRI and NotI. This plasmid was named "pcDNAII/L3 3' 200bp pro." The primer 10 sequences were as follows:

L3-BOT(-1)	GGTAGGCTAGCAGAGCGAGCT
KS primer	TCGAGGGTCGACGGTATC

15            The remainder of the L3 promoter region was isolated from the 5 kb sub-cloned fragment designated 28B 2-3. By restriction mapping, a 600 bp NotI/Clal fragment was identified that would provide a contiguous promoter when combined with the 200bp promoter region. This 600 bp fragment was cloned into pBSK (Stratagene, La Jolla, CA) cleaved with NotI and Clal (clone pBSK/L3 5' 600bp pro). 20            To combine this fragment with "pcDNAII/L3 3' 200bp pro", the 600 bp promoter fragment from "pBSK/L3 5' 600bp pro" was removed using NotI and Xhol and then cloned into "pcDNAII/L3 3' 200bp pro" cleaved with NotI and Xhol. A representative clone from this ligation containing the full 800 bp assembled promoter was designated "L3 pcDNAII contiguous."

25

          To obtain additional sequence of the 5' promoter region, a sequencing primer was designed on the bottom strand of clone 28B 2-3 near the Clal site (approximately 800 bp upstream of ATG) (L3 ClalB; SEQ ID NO:17). An additional 300 bp of nucleotide sequence was obtained, which identified a unique ScaI site upstream. This 30 Clal / ScaI fragment was isolated from 28B 2-3 and sub-cloned into "L3 pcDNAII contiguous" cleaved with Clal and HindII. A full length promoter was assembled

sequence was sequenced by the double-stranded dideoxy termination method using the Sequenase Sequencing Kit (US Biochemicals), according to the manufacturer's instructions. DNA sequencing reactions were analyzed by standard protocols (Sambrook *et al.*, 1989), utilizing gel electrophoresis, followed by detection of 5 radioactive sequencing products by exposure to X-ray film.

Approximately 30 ng of amplified PCR product was labeled with  $^{32}\text{P}$ -dCTP using Pharmacia Random-Priming Kit (Pharmacia, Piscataway, NJ), according to the manufacturer's instructions, and used to probe 30,000 genomic clones from a M017 10 genomic library (Stratagene, La Jolla, CA) on duplicate filters using standard protocols (Sambrook *et al.*, 1989). Four clones were picked from the L3 screen. Individual clones were further purified by plating of 1:500 dilutions and subsequent screening. Four clones were purified to homogeneity, designated 28B, 29B, 32B, 35B.

15

Bacteriophage  $\lambda$  DNA was prepared from the 28B clone by the standard plate lysate method (Sambrook *et al.*, 1989). DNA from clone 28B was restricted with NotI enzyme and subjected to Southern blot analysis using the 300 bp oleosin sequence prepared as described above. The digested genomic clone produced 11 and 5 kb 20 fragments, and the 11 kb fragment hybridized with the 300 bp oleosin probe. Both NotI fragments were isolated and cloned into NotI digested pBSK (Stratagene, La Jolla, CA). These subclones were designated 28B 1-8 (11 kb insert) and 28B 2-3 (5 kb insert) and each was subjected to nucleotide sequence analysis as previously described. Clone 28B 1-8 contained the oleosin coding sequence and approximately 25 200 bp of 5' flanking sequence. Clone 28B 2-3 contained 5' sequence upstream of that present in clone 28B 1-8.

The 28B 1-8 clone was used as PCR template for amplification of promoter fragments. PCR primers L3-BOT(-1) (SEQ ID NO:15) and the vector localized KS 30 primer (SEQ ID NO:16) were used to PCR amplify the region of the genomic clone which is approximately 200 bp upstream of the ATG start codon (PCR amplification conditions used as described previously). The PCR amplification yielded a 250 bp

sequence complementary to nucleotides 407-427. The predicted PCR amplification product is 305 bp. The primer sequences were as follows:

5      L3-TOP                    CGGGTCGATGCTGGTGCTGTC  
          L3-BOT                    GGTGATGCGGTGCTGTGCTG

The conditions for PCR amplification of the oleosin coding sequence were as follows:

10     20 % v/v glycerol  
          1X PCR Buffer (Supplied with Taq Polymerase, Perkin-Elmer Corp., Foster  
          City, CA)  
          1.5 mM MgCl<sub>2</sub>  
          0.2 mM dNTPs  
          0.5 mM each oligonucleotide  
15     1  $\mu$ L genomic DNAs (100 ng of Mo17 genomic DNA)  
          6.5 units Taq polymerase (Perkin-Elmer Corp., Foster City, CA)

The thermocycling profile for amplification was:

20                            1. 94°C            2 minutes  
                          2. 94°C            1 minute  
                          3. 62°C            1 minute  
                          4. 72°C            1 minute  
                          5. 36 cycles to step 2  
                          6. 72°C            4 minutes  
25                            7. 4°C            hold

DNA bands of predicted sizes (approximately 300 bp) were purified by gel excision from a 2% w/v agarose gel and further purified using the Elu-Quick kit (Schelicher and Schuell, Keene, NH) according to manufacturer's instructions.  
30    Isolated DNA fragments were ligated into plasmid pDK101 (ATCC 77406) which had been cleaved with XcmI using the Boehringer-Mannheim (Indianapolis, IN) Rapid Ligation Kit, according to the manufacturer's instructions. The cloned oleosin coding

contain the EGFP::NPTII sequence in the appropriate orientation relative to the promoter sequence.

## EXAMPLE 2

5

### Isolation of the L3 Promoter

Plants from the inbred maize line Mo17 were propagated in the greenhouse and grown for several weeks for the isolation of genomic DNA. Genomic DNA was prepared from 2-3 week old leaf material according to the following protocol. Frozen leaf tissue (2 grams fresh weight) was ground into a fine powder with a glass rod under liquid nitrogen. Powdered tissue was mixed thoroughly with 8 ml of extraction buffer (100 mM Tris, pH 8.0; 50 mM EDTA; 1 % v/v SDS; 500 mM NaCl), pre-warmed to 60°C, followed by a 45 minute incubation at 60°C. The sample was then mixed with 2.5 ml of ice-cold 5 M potassium acetate and then incubated on ice for 20 minutes. Protein aggregates were removed by centrifugation at 3750 rpm for 20 minutes. The supernatant was poured through a layer of Miracloth and the DNA was precipitated by mixing with 5 ml of isopropyl alcohol. Precipitated DNA was collected by centrifugation at 3750 rpm for 15 minutes. The supernatant was poured off of the pelleted DNA and the tube was inverted for 5 minutes to allow residual supernatant to drain from the pellet. DNA was resuspended in 300 µL of water containing 50 mM Tris, pH 8.0, 10 mM EDTA and 3 µL of RNase (10 mg/mL stock). The DNA was precipitated again by the mixing with 50 µL of 4.4 M ammonium acetate, pH 5.2, and 350 µL of isopropyl alcohol, followed by centrifugation in a microcentrifuge at 14,000 rpm for 10 minutes. The DNA pellet was washed with 750 µL of 80 % v/v ethanol and then allowed to drain by inversion for 10 minutes. The DNA was resuspended in 200 µL of water containing 10 mM Tris, pH 8.0, and 1 mM EDTA.

The genomic DNA was used as a template for PCR reactions which utilized PCR primers derived from a maize oleosin sequence (L3 mRNA partial coding sequence.; Locus MZEMPL3; GenBank accession M17225). Oligo L3-TOP (SEQ ID NO:13) matches nucleotides 123-143 and L3-BOT (SEQ ID NO:14) matches the

NcoI and NotI, which provides a diagnostic set of fragments of 738 bp, 891 bp, 1468 bp, and 3681 bp for those clones which contain the MGFP::NPTII sequence in the appropriate orientation relative to the promoter sequence.

5 To construct DV131 (containing an L3/EGFP::NPTII/Tr7 expression cassette; SEQ ID NO:3), the EGFP:: NPTII fragment was excised from DV126 with EcoRI and HindIII and blunt ends generated by using Klenow polymerase. This was then cloned into the backbone of plasmid pDPG778, which was obtained as follows: pDPG778 was cleaved with EcoRI and SmaI to release the GUS coding sequence from the 10 plasmid backbone containing the L3 promoter and the Tr7 terminator. The backbone of 4501 bp was purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA) and blunt ends generated by using Klenow polymerase. This backbone was ligated to the blunt-ended EGFP::NPTII fragment under standard conditions. Plasmid clones containing the 15 desired orientation of the MGFP::NPTII fragment were identified by cleavage with NcoI and NotI, which provides a diagnostic set of fragments of 199 bp, 891 bp, 1282 bp, and 3682 bp for those clones which contain the MGFP::NPTII sequence in the appropriate orientation relative to the promoter sequence.

20 To construct DV133 (containing an L3/rACTI IntronI/EGFP::NPTII/Tr7 expression cassette; SEQ ID NO:5), the EGFP:: NPTII fragment was excised from DV126 with EcoRI and HindIII and blunt ends generated by using Klenow polymerase. This was then cloned into the backbone of plasmid pDPG779 which was obtained as follows: pDPG779 was cleaved with SmaI to release the GUS coding 25 sequence from the plasmid backbone containing the L3 promoter and the Tr7 terminator. The backbone of 5047 bp was purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA) and ligated to the blunt-ended EGFP::NPTII fusion under standard conditions. Plasmid clones containing the desired orientation of the EGFP::NPTII 30 fragment were identified by cleavage with NcoI and NotI, which provides a diagnostic set of fragments of 740 bp, 891 bp, 1282 bp, and 3682 bp for those clones which

These novel coding sequences were placed under the control of the embryo/aleurone specific mzL3 promoter in the following expression cassettes, which were prepared as described in Example 3:

5 pDPG778 - L3/GUS/Tr7  
pDPG779 - L3/rACTI IntronI/GUS/Tr7

To construct DV130 (containing an L3/MGFP::NPTII/Tr7 expression cassette; SEQ ID NO:2), the MGFP::NPTII fusion was excised from DV127 with EcoRI and HindIII, purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA), and blunt ends were generated by using Klenow polymerase (Boehringer Mannheim, Indianapolis, IN) as described in Sambrook *et al.*, (1989). This fragment was then cloned into pDPG778 which had been cleaved with restriction enzymes EcoRI and SmaI and blunt-ended in the same manner. Plasmid clones containing the desired orientation of the GFP::NPTII fragment were identified by cleavage with NcoI and NotI, which provides a diagnostic set of fragments of 197 bp, 891 bp, 1468 bp, and 3681 bp for those clones which contain the MGFP::NPTII sequence in the appropriate orientation relative to the promoter sequence.

20

To construct DV132 (containing an L3/rACTI IntronI/MGFP::NPTII/Tr7 expression cassette; SEQ ID NO:4), the MGFP:: NPTII fusion was excised from DV127 with EcoRI and HindIII, purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA) and blunt ends generated by using Klenow polymerase. This was then cloned into the backbone of plasmid pDPG779, which was obtained as follows: pDPG779 was cleaved with SmaI to release a GUS coding sequence from the plasmid backbone containing the L3 promoter and the Tr7 terminator. The backbone of 5047 bp plasmid was purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA) and ligated to the blunt-ended MGFP::NPTII fusion under standard conditions. Plasmid clones containing the desired orientation of the MGFP::NPTII fragment were identified by cleavage with

plasmid cloning vector pCR2.1 (Invitrogen; Carlsbad, CA) in a reaction containing T4 DNA ligase from Boehringer Mannheim (Indianapolis, IN) using the reaction buffer provided by the manufacturer. The clone containing the EGFP amplification product was designated DV123.

5

The NPTII protein coding sequence was amplified by using a similar strategy to that above, with the exception that the start codon was excluded from the 5' primer and the stop codon was included in the 3' primer. Primers corresponded to nucleotide position 528-548 (NEO5' BAM; SEQ ID NO:11) or positions 1299-1316 (NEO3' 10 SAC; SEQ ID NO:12) of the NPTII sequence contained in pDPG832. These primers contained either an additional BamHI "tail" (NEO5' BAM) or SacI "tail" (NEO3' SAC). Amplification and cycling conditions were identical to those used for the EGFP reaction described above but using 100pg pDPG 832 as template. The expected 805bp band was purified by gel electrophoresis, band excision, and DNA 15 elution by using the components of the Genelute kit (Supelco; Bellafonte, PA) and cloned into pCR2.1 as described above. This clone was designated DV125. The primer sequences were as follows:

NEO5' BAM - CGGGATCCATTGAACAAAGATTGCAC  
20 NEO3' SAC - GGGAGCTCTCAGAAGAACTCGTCAAGAAG

To create the desired fusion protein, DV125 was cleaved with BamHI and SacI to separate the NPTII coding sequence from the remainder of the plasmid and the 802 bp fragment containing the NPTII coding sequence was purified by gel 25 electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA). DV123 and DV124 were cleaved with these same enzymes, creating complementary cloning sites that would join the GFP and NPTII coding sequences at the BamHI site. These ligations were performed using standard procedures and the clones obtained have been named DV127 (MGFP::NPTII 30 in pCR2.1) and DV134 (EGFP::NPTII in pCR2.1).

Amplification was performed by using a Robocycler apparatus (Stratagene, La Jolla, CA) programmed as follows:

95°	- 2 min.	1 cycle
94°	- 1 min.	
5 56-67° gradient	- 1 min.	
72°	- 1 min.	32 cycles
72°	- 4 min.	1 cycle
6°	- hold	

10 PCR amplification yielded the expected 915bp fragment, which was purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA) and ligated into the plasmid cloning vector pCR2.1 (Invitrogen; Carlsbad, CA) in a reaction containing T4 DNA ligase from Boehringer Mannheim (Indianapolis, IN) using the reaction buffer provided by the manufacturer. The clone containing the MGFP amplification product was designated DV124.

20 Another GFP sequence was obtained from the plasmid EGFP (Clontech: Palo Alto, CA; Catalog No. 6077-1). Primers were designed using same strategy as above, including the translation initiation codon in the 5' primer (FP 5' NCO2; SEQ ID NO:9) and excluding the stop codon in the 3' primer (FP 3' BAM; SEQ ID NO:10):

FP 5' NCO2 - CCGGCCATGGTGAGCAAGGGCG

FP 3' BAM - CTGGATCCCTTGTACAGCTCGTCCATGC

25

PCR amplification conditions for the EGFP sequence, carried out using the Clontech EGFP plasmid as template and the primers "FP 5' NCO2" and "FP 3' BAM," were identical to those described above for the MGFP sequence.

30

PCR amplification yielded the expected 731 bp fragment, which was subsequently purified by gel electrophoresis, band excision, and DNA elution by using the components of the Genelute kit (Supelco; Bellafonte, PA) and ligated into the

made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### EXAMPLE 1

5

#### Generation of GFP::NPTII Fusion Protein Constructs

Protein coding sequences for each of two green fluorescent proteins (GFPs) were individually fused to the neomycin phosphotransferase (NPTII) protein coding sequence by a cloning strategy which allowed for generation of in-frame translational fusions. The preparation of these constructs was as follows. Primers were designed 10 for amplification of a GFP sequence, herein designated as MGFP, reported by Pang *et al.* (Plant Phys. 112:893-900, 1996). These primers correspond to nucleotide positions 16-32 (M-FP 5'NCO; SEQ ID NO:7), including the translation initiation codon, and nucleotide positions 903-922 (M-FP 3'BAM; SEQ ID NO:8), excluding 15 the stop codon, of the above reported sequence. This strategy allowed for the addition of NcoI and BamHI restriction sites to facilitate subsequent cloning manipulation steps. The sequence of the primers was as given below:

M-FP 5'NCO AACCATGGCAAGGGCG

M-FP 3'BAM CTGGATCCCTGTAGAGTTCATCCATGC

20

The plasmid pMON30107 (Pang *et al.*, 1996) was used as template for PCR amplification with these primers by using the following conditions with a HF PCR Kit (Boehringer Mannheim; Indianapolis, IN).

25 100pg template

200μM dATP

200μM dCTP

200μM dGTP

200μM dTTP

30 500nM each primer

3.75mM MgCl<sub>2</sub>

**Transformed cell:** A cell whose DNA complement has been altered by the introduction of an exogenous DNA molecule into that cell.

**Transgene:** A segment of DNA which is incorporated into a host genome or is capable of autonomous replication within a host cell and is capable of causing the expression of one or more cellular products. Exemplary transgenes will provide the host cell, or plants regenerated therefrom, with a novel phenotype relative to the corresponding non-transformed cell or plant.

**Transgenic cell:** Any cell derived or regenerated from a transformed cell and comprising a transgene. Exemplary transgenic cells include plant calli derived from a transformed plant cell and particular cells such as leaf, root, stem, e.g., somatic cells, or reproductive (germ) cells obtained from a transgenic plant.

**Transgenic plant:** A plant or progeny of any subsequent generation derived therefrom, of a transformed plant cell or protoplast comprising a transgene, wherein the transgene comprises an introduced exogenous DNA molecule not originally present in a native, non-transgenic plant of the same strain. The transgenic plant may additionally contain sequences which are native to the plant being transformed, but wherein the "exogenous" gene has been altered by gene technological means in order to alter the level or pattern of expression of the gene.

**Transit Peptide:** A polypeptide sequence which is capable of directing a polypeptide to a particular organelle or other location within a cell.

**Vector:** A DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

**25 XIII. Examples**

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be

**Automated:** When used in the context of seed selection or screening, this term refers to the use of an apparatus or machine for the identification and determination of seeds which express a given screenable marker.

**Exogenous gene:** A gene which is not naturally found in the host genome. In this respect, the gene itself may be native to the host genome, however, the exogenous gene will have been modified by the addition or deletion of one or more different regulatory or other elements.

**Expression:** The combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule in the production of a polypeptide.

**Marker Gene:** A gene which confers one or more recognizable phenotypes upon a plant cell, plant, plant part, or seed.

**Progeny:** Any subsequent generation, including the seeds and plants therefrom, which is derived from a particular parental plant or set of parental plants.

**Promoter:** A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a structural gene or gene fusion and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

**Regeneration:** The process of growing a plant from a plant cell (e.g., plant protoplast or explant).

**Screenable marker gene:** A gene which encodes a cellular product providing a cell, cell cluster, plant, plant part or seed with a recognizable phenotype.

**Selectable marker gene:** A gene which encodes a cellular product that provides a cell, cell cluster, plant, plant part or seed with resistance to one or more selective agents, for example, a herbicide or antibiotic.

**Selectable-screenable marker gene fusion:** A gene fusion between a selectable and a screenable marker gene, the polypeptide product of which comprises both a screenable and a selectable marker gene function.

**Transformation:** A process of introducing an exogenous DNA sequence or construct (e.g., a vector or expression cassette) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

recombination sequences is one approach to remedying this situation. The site-specific recombination sequence can be mutated in a manner that the product of the recombination reaction is no longer recognized as a substrate for the reverse reaction, thereby stabilizing the integration or excision event.

5

In the Cre-lox system, discovered in bacteriophage P1, recombination between loxP sites occurs in the presence of the Cre recombinase (see, e.g., U.S. Patent No. 5,658,772, specifically incorporated herein by reference in its entirety). This system has been utilized to excise a gene located between two lox sites which had been 10 introduced into a yeast genome (Sauer, 1987). Cre was expressed from an inducible yeast GAL1 promoter and this Cre gene was located on an autonomously replicating yeast vector.

Since the lox site is an asymmetrical nucleotide sequence, lox sites on the 15 same DNA molecule can have the same or opposite orientation with respect to each other. Recombination between lox sites in the same orientation results in a deletion of the DNA Segment located between the two lox sites and a connection between the resulting ends of the original DNA molecule. The deleted DNA segment forms a circular molecule of DNA. The original DNA molecule and the resulting circular 20 molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA molecules can occur. All of these recombination events are catalyzed by the 25 product of the Cre coding region.

### XIII. Definitions

**Aleurone-specific promoter:** A promoter which directs the expression of a gene preferentially in the aleurone and/or embryo of a plant seed. By "preferentially," 30 it is meant a promoter which expresses a screenable marker gene at least a 50% greater level in the aleurone of a seed than in vegetative tissue or in endosperm tissue.

will also likely contain numerous copies of randomly integrated introduced DNA sequences. Therefore, to maintain control over the copy number and the location of the inserted DNA, these randomly inserted DNA sequences can be removed. One manner of removing these random insertions is to utilize a site-specific recombinase system. In general, a site specific recombinase system consists of three elements: two pairs of DNA sequence (the site - specific recombination sequences) and a specific enzyme (the site-specific recombinase). The site-specific recombinase will catalyze a recombination reaction only between two site -specific recombination sequences.

10 A number of different site specific recombinase systems could be employed in accordance with the instant invention, including, but not limited to, the Cre/lox system of bacteriophage P1 (U.S. Patent No. 5,658,772, specifically incorporated herein by reference in its entirety), the FLP/FRT system of yeast (Golic and Lindquist, 1989), the Gin recombinase of phage Mu (Maeser *et al.*, 1991), the Pin recombinase of *E. coli* (Enomoto *et al.*, 1983), and the R/RS system of the pSR1 plasmid (Araki *et al.*, 1992). The bacteriophage P1 Cre/lox and the yeast FLP/FRT systems constitute two particularly useful systems for site specific integration or excision of transgenes. In these systems a recombinase (Cre or FLP) will interact specifically with its respective site -specific recombination sequence (lox or FRT, respectively) to invert or excise the intervening sequences. The sequence for each of these two systems is relatively short (34 bp for lox and 47 bp for FRT) and therefore, convenient for use with transformation vectors.

25 The FLP/FRT recombinase system has been demonstrated to function efficiently in plant cells. Experiments on the performance of the FLP/FRT system in both maize and rice protoplasts indicate that FRT site structure, and amount of the FLP protein present, affects excision activity. In general, short incomplete FRT sites leads to higher accumulation of excision products than the complete full-length FRT sites. The systems can catalyze both intra- and intermolecular reactions in maize protoplasts, indicating its utility for DNA excision as well as integration reactions. 30 The recombination reaction is reversible and this reversibility can compromise the efficiency of the reaction in each direction. Altering the structure of the site - specific

sequences contained on the introduced DNA will determine the site where the introduced DNA is integrated via homologous recombination. For example, if the DNA sequence of interest is linked to DNA sequences sharing homology to a single copy gene of a host plant cell, the DNA sequence of interest will be inserted via 5 homologous recombination at only that single specific site. However, if the DNA sequence of interest is linked to DNA sequences sharing homology to a multicopy gene of the host eukaryotic cell, then the DNA sequence of interest can be inserted via homologous recombination at each of the specific sites where a copy of the gene is located.

10

DNA can be inserted into the host genome by a homologous recombination reaction involving either a single reciprocal recombination (resulting in the insertion of the entire length of the introduced DNA) or through a double reciprocal recombination (resulting in the insertion of only the DNA located between the two 15 recombination events). For example, if one wishes to insert a foreign gene into the genomic site where a selected gene is located, the introduced DNA should contain sequences homologous to the selected gene. A single homologous recombination event would then result in the entire introduced DNA sequence being inserted into the selected gene. Alternatively, a double recombination event can be achieved by 20 flanking each end of the DNA sequence of interest (the sequence intended to be inserted into the genome) with DNA sequences homologous to the selected gene. A homologous recombination event involving each of the homologous flanking regions will result in the insertion of the foreign DNA. Thus only those DNA sequences located between the two regions sharing genomic homology become integrated into 25 the genome.

Although introduced sequences can be targeted for insertion into a specific genomic site via homologous recombination, in higher eukaryotes homologous recombination is a relatively rare event compared to random insertion events. In plant 30 cells, foreign DNA molecules find homologous sequences in the cell's genome and recombine at a frequency of approximately  $0.5\text{--}4.2 \times 10^{-4}$ . Thus any transformed cell that contains an introduced DNA sequence integrated via homologous recombination

## XII. Site Specific Integration or Excision of Transgenes

It is specifically contemplated by the inventors that one could employ techniques for the site-specific integration or excision of transformation constructs prepared in accordance with the instant invention. An advantage of site-specific integration or excision is that it can be used to overcome problems associated with conventional transformation techniques, in which transformation constructs typically randomly integrate into a host genome in multiple copies. This random insertion of introduced DNA into the genome of host cells can be lethal if the foreign DNA inserts into an essential gene. In addition, the expression of a transgene may be influenced by "position effects" caused by the surrounding genomic DNA. Further, because of difficulties associated with plants possessing multiple transgene copies, including gene silencing, recombination and unpredictable inheritance, it is typically desirable to control the copy number of the inserted DNA, often only desiring the insertion of a single copy of the DNA sequence.

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Site-specific integration or excision of transgenes or parts of transgenes can be achieved in plants by means of homologous recombination (see, for example, U.S. Patent No. 5,527,695, specifically incorporated herein by reference in its entirety). Homologous recombination is a reaction between any pair of DNA sequences having a similar sequence of nucleotides, where the two sequences interact (recombine) to form a new recombinant DNA species. The frequency of homologous recombination increases as the length of the shared nucleotide DNA sequences increases, and is higher with linearized plasmid molecules than with circularized plasmid molecules. Homologous recombination can occur between two DNA sequences that are less than identical, but the recombination frequency declines as the divergence between the two sequences increases.

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Introduced DNA sequences can be targeted via homologous recombination by linking a DNA molecule of interest to sequences sharing homology with endogenous sequences of the host cell. Once the DNA enters the cell, the two homologous sequences can interact to insert the introduced DNA at the site where the homologous genomic DNA sequences were located. Therefore, the choice of homologous

Other studies may be performed using a more conventional relative quantitative RT-PCR assay with an external standard protocol. These assays sample the PCR products in the linear portion of their amplification curves. The number of PCR cycles that are optimal for sampling must be empirically determined for each target cDNA fragment. In addition, the reverse transcriptase products of each RNA population isolated from the various tissue samples must be carefully normalized for equal concentrations of amplifiable cDNAs. This consideration is very important since the assay measures absolute mRNA abundance. Absolute mRNA abundance can be used as a measure of differential gene expression only in normalized samples.

5 While empirical determination of the linear range of the amplification curve and normalization of cDNA preparations are tedious and time consuming processes, the resulting RT-PCR assays can be superior to those derived from the relative quantitative RT-PCR assay with an internal standard.

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15 One reason for this advantage is that without the internal standard/competitor, all of the reagents can be converted into a single PCR product in the linear range of the amplification curve, thus increasing the sensitivity of the assay. Another reason is that with only one PCR product, display of the product on an electrophoretic gel or another display method becomes less complex, has less background and is easier to interpret.

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(vii) *Chip Technologies*

Specifically contemplated by the present inventors are chip-based DNA technologies such as those described by Hacia *et al.* (1996) and Shoemaker *et al.* 25 (1996). Briefly, these techniques involve quantitative methods for analyzing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ chip technology to segregate target molecules as high density arrays and screen these molecules on the basis of hybridization (see also, Pease *et al.*, 1994; and Fodor *et al.*, 1991).

5        The second condition that must be met for an RT-PCR experiment to successfully determine the relative abundances of a particular mRNA species is that relative concentrations of the amplifiable cDNAs must be normalized to some independent standard. The goal of an RT-PCR experiment is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample.

10      Most protocols for competitive PCR utilize internal PCR standards that are approximately as abundant as the target. These strategies are effective if the products of the PCR amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundances made for many different RNA samples, such as is the case when examining RNA 15     samples for differential expression, become distorted in such a way as to make differences in relative abundances of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant than the target. If the internal standard is more abundant than the target, then direct linear comparisons can be made between RNA samples:

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      The above discussion describes theoretical considerations for an RT-PCR assay for plant tissue. The problems inherent in plant tissue samples are that they are of variable quantity (making normalization problematic), and that they are of variable quality (necessitating the co-amplification of a reliable internal control, preferably of 25     larger size than the target). Both of these problems are overcome if the RT-PCR is performed as a relative quantitative RT-PCR with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal standard is roughly 5-100 fold higher than the mRNA encoding the target. This assay measures 30     relative abundance, not absolute abundance of the respective mRNA species.

In PCR, the number of molecules of the amplified target DNA increase by a factor approaching two with every cycle of the reaction until some reagent becomes limiting. Thereafter, the rate of amplification becomes increasingly diminished until 5 there is no increase in the amplified target between cycles. If a graph is plotted in which the cycle number is on the X axis and the log of the concentration of the amplified target DNA is on the Y axis, a curved line of characteristic shape is formed by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After a 10 reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

15 The concentration of the target DNA in the linear portion of the PCR amplification is directly proportional to the starting concentration of the target before the reaction began. By determining the concentration of the amplified products of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are 20 cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundances of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCR products and the relative mRNA abundances is only true in the linear range of the PCR reaction.

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The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. Therefore, the first condition that must be met before the relative abundances of a mRNA species can be determined by RT-PCR for 30 a collection of RNA populations is that the concentrations of the amplified PCR products must be sampled when the PCR reactions are in the linear portion of their curves.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by a labeled probe. The techniques involved are well known to those of skill in the art and can be found in many standard books on molecular protocols (see Sambrook *et al.*, 1989). For example, chromophore or radiolabel probes or primers identify the target during or following amplification.

One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

In addition, the amplification products described above may be subjected to sequence analysis to identify specific kinds of variations using standard sequence analysis techniques. Within certain methods, exhaustive analysis of genes is carried out by sequence analysis using primer sets designed for optimal sequencing (Pignon *et al.*, 1994). The present invention provides methods by which any or all of these types of analyses may be used.

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(vi) *Design and Theoretical Considerations for Relative Quantitative RT-PCR*

Reverse transcription (RT) of RNA to cDNA followed by relative quantitative PCR (RT-PCR) can be used to determine the relative concentrations of specific mRNA species isolated from plants. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed.

Briefly, a probe is used to target a DNA or RNA species that has been immobilized on a suitable matrix, often a filter of nitrocellulose. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by "blotting" on to the filter.

5

Subsequently, the blotted target is incubated with a probe (usually labeled) under conditions that promote denaturation and rehybridization. Because the probe is designed to base pair with the target, the probe will bind a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection 10 is accomplished as described above.

(iv) *Separation Methods*

It normally is desirable, at one stage or another, to separate the amplification product from the template and the excess primer for the purpose of determining 15 whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.*, 1989.

Alternatively, chromatographic techniques may be employed to effect 20 separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography (Freifelder, 1982).

25

(v) *Detection Methods*

Products may be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the 30 amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

"Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

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Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, M.A., In: *PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS*, Academic Press, N.Y., 1990; Ohara *et al.*, 1989; each incorporated herein by reference in their entirety).

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Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, also may be used in the amplification step of the present invention. Wu *et al.*, (1989), incorporated herein by reference in its entirety.

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(iii) *Southern/Northern Blotting*

Blotting techniques are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA as a target. Each provide different types of information, although cDNA blotting is analogous, in many aspects, to blotting of RNA species.

the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based  
5 amplification systems (TAS), including nucleic acid sequence based amplification  
(NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO  
88/10315, incorporated herein by reference in their entirety). In NASBA, the nucleic  
10 acids can be prepared for amplification by standard phenol/chloroform extraction, heat  
denaturation of a clinical sample, treatment with lysis buffer and minispin columns for  
isolation of DNA and RNA or guanidinium chloride extraction of RNA. These  
15 amplification techniques involve annealing a primer which has target specific  
sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H  
while double stranded DNA molecules are heat denatured again. In either case, the  
single stranded DNA is made fully double stranded by addition of second target  
20 specific primer, followed by polymerization. The double-stranded DNA molecules  
are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an  
isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded  
DNA, which is then converted to double stranded DNA, and then transcribed once  
again with an RNA polymerase such as T7 or SP6. The resulting products, whether  
truncated or complete, indicate target specific sequences.

Davey *et al.*, EPO No. 329 822 (incorporated herein by reference in its  
entirety) disclose a nucleic acid amplification process involving cyclically  
synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA  
25 (dsDNA), which may be used in accordance with the present invention. The ssRNA  
is a template for a first primer oligonucleotide, which is elongated by reverse  
transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from  
the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase  
specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a  
30 template for a second primer, which also includes the sequences of an RNA  
polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the  
template. This primer is then extended by DNA polymerase (exemplified by the large

target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

5 An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site also may be useful in the amplification of nucleic acids in the present invention, Walker *et al.*, (1992).

10 Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four 15 bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences also can be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with 20 RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

25 Still another amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling 30 with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage,

Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases and are described in WO 90/07641, filed December 21, 1990. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent No. 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, also may be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a

transformed plants. In this way, it is possible to detect differences in the amount or kind of protein detected in various transformed plants.

5 A variety of different assays are contemplated in the screening of transgenic plants created using the methods of the current invention. These techniques can be used to detect for both the presence of particular genes as well as rearrangements that may have occurred in the gene construct. The techniques include but are not limited to, fluorescent *in situ* hybridization (FISH), direct DNA sequencing, PFGE analysis, 10 Southern or Northern blotting, single-stranded conformation analysis (SSCA), RNase protection assay, allele-specific oligonucleotide (ASO), dot blot analysis, denaturing gradient gel electrophoresis, RFLP and PCR-SSCP.

(i) *Primers and Probes*

15 The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred. Probes are defined differently, although they may act as primers. Probes, 20 while perhaps capable of priming, are designed to bind to the target DNA or RNA and need not be used in an amplification process.

25 In preferred embodiments, the probes or primers are labeled with radioactive species ( $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ , or other label), with a fluorophore (rhodamine, fluorescein), an antigen (biotin, streptavidin, digoxigenin), or a chemiluminescent (luciferase).

(ii) *Template Dependent Amplification Methods*

30 A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR<sup>TM</sup>) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in its entirety.

germinated directly on an appropriate media. Smaller embryos may be cultured for one week on media containing the above ingredients along with  $10^{-5}$ M abscisic acid and then transferred to growth regulator-free medium for germination.

5        **XI.    Genetic Analysis of Transgenic Plants**

In particular embodiments of the invention, molecular genetic techniques may be used for detecting the presence or expression of transgenes in plants. For example, the expression of a transgene construct in a particular tissue could be confirmed by performing of assays. The method of assaying may comprise determining the level of protein expressed by the transgene or by determining specific alterations in the expressed product. Such assays may in some cases be faster, more accurate or less expensive than conventional screening assays.

15        The biological sample may potentially be any type of plant tissue. Nucleic acid may be isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA; in another, it is poly-A RNA. Normally, the nucleic acid is amplified.

20        Depending on the format, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (*e.g.*, ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Affymax Technology).

30        Following detection, one may compare the results seen in a given plant with a statistically significant reference group of non-transformed control plants. Typically, the non-transformed control plants will be of a genetic background similar to the

preferred growth regulator for such purposes is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or perhaps even picloram. Media improvement in these and similar ways have been found to facilitate the growth of cells at specific developmental stages. Tissue may be maintained on a 5 basic media with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of manual selection until the morphology of the tissue is suitable for regeneration, at least two weeks, then transferred to media conducive to maturation of embryoids. Cultures are transferred about every two weeks on this medium, although the time between subcultures may 10 vary depending on the rate of growth of the cells. For example, cultures may be transferred at 3 week intervals. Shoot development will signal the time to transfer to medium lacking growth regulators.

15 The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, then will be allowed to mature into plants. Developing plantlets are transferred to soilless plant growth mix, and hardened, *e.g.*, in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO<sub>2</sub>, and 25-250 microeinsteins m<sup>-2</sup> s<sup>-1</sup> of light. Plants preferably are matured either in a growth chamber or greenhouse. Plants are regenerated from 20 about 6 weeks to 10 months after a transformant is identified, depending on the initial tissue. During regeneration, cells are grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are petri dishes, Plant Cons and Phytatrays. Regenerating plants preferably are grown at about 19°C to 28°C. After the 25 regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

It should be noted, however, that kernels on transformed plants may occasionally require embryo rescue due to cessation of kernel development and premature senescence of plants. To rescue developing embryos, they are excised from 30 surface-disinfected kernels 10-20 days post-pollination and cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In embryo rescue, large embryos (defined as greater than 3 mm in length) are

glyphosate resistant EPSPS enzymes are described in, for example, International Patent WO 97/4103. The best characterized mutant EPSPS gene conferring glyphosate resistance comprises amino acid changes at residues 102 and 106, although it is anticipated that other mutations will also be useful (PCT/WO97/4103).

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It is further contemplated that the herbicide DALAPON, 2,2-dichloropropionic acid, may be useful for identification of transformed cells. The enzyme 2,2-dichloropropionic acid dehalogenase (*deh*) inactivates the herbicidal activity of 2,2-dichloropropionic acid and therefore confers herbicidal resistance on cells or plants expressing a gene encoding the dehalogenase enzyme (Buchanan-Wollaston *et al.*, 1992; U.S. Patent Appl. No. 08/113,561, filed August 25, 1993; U.S. Patent No. 5,508,468; U.S. Patent No. 5,508,468).

15 Alternatively, a gene encoding an anthranilate synthase gene which confers resistance to certain amino acid analogs, *e.g.*, 5-methyltryptophan or 6-methyl anthranilate, may be useful as a selectable marker gene. The use of an anthranilate synthase gene as a selectable marker was described in U.S. Patent No. 5,508,468; and U.S. Patent Serial No. 08/604,789.

20 The *neo* gene, encoding the NPTII protein, is particularly useful for selection of transformed plant cells. The *neo* gene was isolated from the transposon Tn5 of *E. coli* and encodes a protein consisting of 264 amino acids (Beck *et al.*, 1982). Expression of the *neo* gene in plant cells confers resistance to amino glycoside antibiotics (such as kanamycin, paromomycin, and G418) by phosphorylation of the 3' 25 hydroxyl group of the antibiotics (Bryan, 1984). The *neo* gene has been successfully used as a selectable marker for transformation of a wide range of plant species.

(ii) *Regeneration and Seed Production*

30 Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In an exemplary embodiment, MS and N6 media may be modified (see Table 8) by including further substances such as growth regulators. A

A herbicide which has been used successfully as a selection agent is the broad spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine residues by intracellular peptidases, the PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, 1973). Synthetic PPT, the active ingredient in the herbicide Liberty™ also is effective as a selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

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The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in DE 3642 829 A, wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism, this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.*, 1987). The *bar* gene has been cloned (Murakami *et al.*, 1986; Thompson *et al.*, 1987) and expressed in transgenic tobacco, tomato, potato (De Block, 1987) and *Brassica* (De Block, 1989) plants. In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial formulations of PPT and bialaphos in greenhouses.

15

Another example of a herbicide which can be used for selection of transformed cell lines is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived thereof. U.S. Patent No. 4,535,060 describes the isolation of EPSPS mutations which confer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, *aroA*. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate resistant *aroA* gene were introduced *in vitro*. Mutant genes encoding

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identification of transformed cells, while the screenable marker system offers an efficient means for selection of seeds which have inherited the transgene construct. It is specifically contemplated by the inventors, however, that cells, clusters of cells (for example, callus or immature embryos), or plants could be selected with either the 5 selectable or screenable marker functions of the constructs of the instant invention. For instance, transformed cells could be identified which express GFP, and transformed plants could be selected by treatment with a selection agent such as paromomycin.

10 (i) *Selection*

It is believed that DNA is introduced into only a small percentage of target cells in any one experiment. In order to provide an efficient system for identification of those cells receiving DNA and integrating it into their genomes one may employ a means for selecting those cells that are stably transformed. In a preferred embodiment 15 of the invention, cells are transformed with a gene fusion between a selectable and a screenable marker gene which encodes a fusion protein having selectable and screenable marker gene activities, and the transformed cells are selected by exposure to appropriate selection agent. One exemplary embodiment of a selectable marker is a gene which confers resistance to some normally inhibitory agent, such as an antibiotic or herbicide. In particular, genes conferring resistance to aminoglycoside antibiotics, 20 for example, neomycin phosphotransferase II (NPT II) or NPTI, are deemed especially useful in this respect. Examples of aminoglycoside antibiotics which may be used for selection with these genes include neomycin, kanamycin and paromomycin. Where NPTII is used with maize cells, paromomycin is preferred for selection.

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To select potentially transformed cells, the cells are first exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable 30 integration of the exogenous DNA. Using the techniques disclosed herein, greater than 40% of bombarded embryos may yield transformants.

free water left in the cells), and they could be safely plunged into liquid nitrogen for storage. The paucity of free water remaining in the cells in conjunction with the rapid cooling rates from -35°C to -196°C prevented large organized ice crystals from forming in the cells. The cells are stored in liquid nitrogen, which effectively immobilizes the cells and slows metabolic processes to the point where long-term storage should not be detrimental.

Thawing of the extracellular solution was accomplished by removing the cryo-tube from liquid nitrogen and swirling it in sterile 42°C water for approximately 2 minutes. The tube was removed from the heat immediately after the last ice crystals had melted to prevent heating the tissue. The cell suspension (still in the cryoprotectant mixture) was pipetted onto a filter, resting on a layer of BMS cells (the feeder layer which provided a nurse effect during recovery). The cryoprotectant solution is removed by pipetting. Culture medium comprised a callus proliferation medium with increased osmotic strength. Dilution of the cryoprotectant occurred slowly as the solutes diffused away through the filter and nutrients diffused upward to the recovering cells. Once subsequent growth of the thawed cells was noted, the growing tissue was transferred to fresh culture medium. If initiation of a suspension culture was desired, the cell clusters were transferred back into liquid suspension medium as soon as sufficient cell mass had been regained (usually within 1 to 2 weeks). Alternatively, cells were cultured on solid callus proliferation medium. After the culture was reestablished in liquid (within 1 to 2 additional weeks), it was used for transformation experiments. When desired, previously cryopreserved cultures may be frozen again for storage.

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#### X. Production and Characterization of Transgenic Plants

After effecting delivery of exogenous DNA to recipient cells, the next steps generally concern identifying the transformed cells for further culturing and plant regeneration. As disclosed herein, in order to improve the ability to identify transformants, the constructs of the invention preferably include selectable and screenable marker gene function, and additionally, one or more expressible gene of interest. The selectable marker gene offers an efficient means for the primary

alternating callus between different culture conditions may be beneficial in enriching for recipient cells within a culture. For example, it is proposed that cells may be cultured in suspension culture, but transferred to solid medium at regular intervals. After a period of growth on solid medium cells can be manually selected for return to liquid culture medium. It is proposed that by repeating this sequence of transfers to fresh culture medium it is possible to enrich for recipient cells. It also is contemplated that passing cell cultures through a 1.9 mm sieve is useful in maintaining the friability of a callus or suspension culture and may be beneficial in enriching for transformable cells.

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(iv) *Cryopreservation Methods*

Cryopreservation is important because it allows one to maintain and preserve a known transformable cell culture for future use, while eliminating the cumulative detrimental effects associated with extended culture periods.

15

Cell suspensions and callus were cryopreserved using modifications of methods previously reported (Finkle, 1985; Withers & King, 1979). The cryopreservation protocol comprised adding a pre-cooled (0°C) concentrated cryoprotectant mixture stepwise over a period of one to two hours to pre-cooled (0°C) cells. The mixture was maintained at 0°C throughout this period. The volume of added cryoprotectant was equal to the initial volume of the cell suspension (1:1 addition), and the final concentration of cryoprotectant additives was 10% dimethyl sulfoxide, 10% polyethylene glycol (6000 MW), 0.23 M proline and 0.23 M glucose. The mixture was allowed to equilibrate at 0°C for 30 minutes, during which time the cell suspension/ cryoprotectant mixture was divided into 1.5 ml aliquot (0.5 ml packed cell volume) in 2 ml polyethylene cryo-vials. The tubes were cooled at 0.5°C/minute to -8°C and held at this temperature for ice nucleation.

30

Once extracellular ice formation had been visually confirmed, the tubes were cooled at 0.5°C/minute from -8°C to -35°C. They were held at this temperature for 45 minutes (to insure uniform freeze-induced dehydration throughout the cell clusters). At this point, the cells had lost the majority of their osmotic volume (*i.e.*, there is little

August 25, 1993, the disclosure of which is specifically incorporated herein by reference.

5 (ii) *Media*

In certain embodiments, recipient cells are selected following growth in culture. Where employed, cultured cells may be grown either on solid supports or in the form of liquid suspensions. In either instance, nutrients may be provided to the cells in the form of media, and environmental conditions controlled. There are many types of tissue culture media comprised of various amino acids, salts, sugars, growth regulators and vitamins. Most of the media employed in the practice of the invention will have some similar components (see, Table 8), but may differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending 10 on the growth media. In some media, cells survive but do not divide.

15 Various types of media suitable for culture of plant cells previously have been described. Examples of these media include, but are not limited to, the N6 medium described by Chu *et al.* (1975) and MS media (Murashige and Skoog, 1962). The inventors have discovered that media such as MS which have a high ammonia/nitrate ratio are counterproductive to the generation of recipient cells in that they promote loss of morphogenic capacity. N6 media, on the other hand, has a somewhat lower ammonia/nitrate ratio, and is contemplated to promote the generation of recipient cells 20 by maintaining cells in a proembryonic state capable of sustained divisions.

25

(iii) *Maintenance*

The method of maintenance of cell cultures may contribute to their utility as 30 sources of recipient cells for transformation. Manual selection of cells for transfer to fresh culture medium, frequency of transfer to fresh culture medium, composition of culture medium, and environment factors including, but not limited to, light quality and quantity and temperature are all important factors in maintaining callus and/or suspension cultures that are useful as sources of recipient cells. It is contemplated that

**Table 8 – Continued**

2004	N6	3%	5.8	1 mg thiamine 0.5 mg niacin 3.3 mg dicamba 17 mg AgNO <sub>3</sub> 1.4 g L-proline 0.8 g L-asparagine 100 mg casein hydrolysate 100 mg myo-inositol Gelrite
2008	N6	3%	5.8	1 mg thiamine 0.5 mg niacin 3.3 mg dicamba 1.4 g L-proline 0.8 g L-asparagine Gelrite

\* Basic MS medium described in Murashige and Skoog (1962). This medium is typically modified by decreasing the  $\text{NH}_4\text{NO}_3$  from 1.64 g/l to 1.55 g/l, and omitting the pyridoxine HCl, nicotinic acid, myo-inositol and glycine.

\*\* NAA = Naphthal Acetic Acid

IAA = Indole Acetic Acid

2-IP = 2, isopentyl adenine

2,4-D = 2, 4-Dichlorophenoxyacetic Acid

BAP = 6-Benzyl aminopurine

ABA = abscisic acid

\*\*\* Basic medium described in Clark (1982)

15 \*\*\*\* These media may be made with or without solidifying agent.

A number of exemplary maize cultures which may be used for transformation have been developed and are disclosed in U.S. Patent Appl. No. 08/113,561, filed

Table 8 – Continued

728	N6	3%	5.8	N6 vitamins 2 mg L-glycine 9.9 mg dicamba 16.9 mg AgNO <sub>3</sub> 100 mg casein hydrolysate 2.9 g L-proline Gelgro
734	N6	2%	5.8	N6 vitamins 2 mg L-glycine 1.5 mg 2,4-D 14 g Fe sequestreene (replaces Fe-EDTA) 200 mg casein hydrolysate 0.69 g L-proline Gelrite
735	N6	2%	5.8	1 mg 2,4-D .5 mg niacin .91 g L-asparagine 100 mg myo-inositol 1 mg thiamine .5 g MES .75 g MgCl <sub>2</sub> 100 mg casein hydrolysate 0.69 g L-proline Gelgro

Table 8 – Continued

721	N6	2%	5.8	3.3 mg dicamba 1 mg thiamine .5 mg niacin 800 mg L-asparagine 100 mg myo-inositol 100 mg casein hydrolysate 1.4 g L-proline 54.65 g mannitol Gelgro
726	N6	3%	5.8	3.3 mg dicamba .5 mg niacin 1 mg thiamine 800 mg L-asparagine 100 mg myo-inositol 100 mg casein hydrolysate 1.4 g L-proline
727	N6	3%	5.8	N6 vitamins 2 mg L-glycine 9.9 mg dicamba 100 mg casein hydrolysate 2.9 g L-proline Gelgro

Table 8 - Continued

409	MS	3%	6.0	3.73 mg Na <sub>2</sub> EDTA .25 mg thiamine 9.9 mg dicamba 200 mg casein hydrolysate 2.9 g L-proline 500 mg K <sub>2</sub> SO <sub>4</sub> 400 mg KH <sub>2</sub> PO <sub>4</sub> 100 mg myo-inositol
501	Clark's Medium***	2%	5.7	
607	1/2 x MS	3%	5.8	1 mg thiamine 1 mg niacin Gelrite
615	MS	3%	6.0	MS vitamins 6 mg BAP 100 mg myo-inositol Bactoagar
617	1/2 x MS	1.5%	6.0	MS vitamins 50 mg myo-inositol Bactoagar
708	N6	2%	5.8	N6 vitamins 2 mg L-glycine 1.5 mg 2,4-D 200 mg casein hydrolysate 0.69 g L-proline Gelrite

Table 8 – Continued

401	MS	3%	6.0	3.73 mg Na <sub>2</sub> EDTA .25 mg thiamine 1 mg 2,4-D 2 mg NAA 200 mg casein hydrolysate 500 mg K <sub>2</sub> SO <sub>4</sub> 400 mg KH <sub>2</sub> PO <sub>4</sub> 100 mg myo- inositol
402	MS	3%	6.0	3.73 mg Na <sub>2</sub> EDTA .25 mg thiamine 1 mg 2,4-D 200 mg casein hydrolysate 2.9 g L- proline 500 mg K <sub>2</sub> SO <sub>4</sub> 400 mg KH <sub>2</sub> PO <sub>4</sub> 100 mg myo- inositol

Table 8 – Continued

273 (also, 201V, 236S, 201D,2071, 2366, 201SV, 2377, and 201BV)	N6	2%	5.8	N6 vitamins 2 mg L- glycine 1 mg 2,4-D 16.9 mg AgNO <sub>3</sub> 100 mg casein hydrolysate 2.9 g L- proline
279	N6	2%	5.8	3.3 mg dicamba 1 mg thiamine .5 mg niacin 800 mg L- asparagine 100 mg casein hydrolysate 100 mg myoinositol 1.4 g L- proline Gelgro****
288	N6	3%		3.3 mg dicamba 1 mg thiamine .5 mg niacin .8 g L- asparagine 100 mg myo- inositol 1.4 g L- proline 100 mg casein hydrolysate 16.9 mg AgNO <sub>3</sub> Gelgro

Table 8 - Continued

210	N6	3%	5.5	N6 vitamins 2 mg 2,4-D 250 mg Ca pantothenate 100 mg myo- inositol 790 mg L- asparagine 100 mg casein hydrolysate 1.4 g L- proline Hazelton agar**** 2 mg L- glycine
212	N6	3%	5.5	N6 vitamins 2 mg L- glycine 2 mg 2,4-D 250 mg Ca pantothenate 100 mg myo- inositol 100 mg casein hydrolysate 1.4 g L- proline Hazelton agar****
227	N6	2%	5.8	N6 vitamins 2 mg L- glycine 13.2 mg dicamba 100 mg casein hydrolysate 2.9 g L- proline Gelgro

Table 8 – Continued

189	MS	-	5.8	3 mg BAP .04 mg NAA .5 mg niacin 800 mg L- asparagine 100 mg casamino acids 20 g sorbitol 1.4 g L- proline 100 mg myo- inositol Gelgro
201	N6	2%	5.8	N6 vitamins 2 mg L- glycine 1 mg 2,4-D 100 mg casein hydrolysate 2.9 g L- proline Gelgro
205	N6	2%	5.8	N6 vitamins 2 mg L- glycine .5 mg 2,4-D 100 mg casein hydrolysate 2.9 g L- proline Gelgro
209	N6	6%	5.8	N6 vitamins 2 mg L- glycine 100 mg casein hydrolysate 0.69 g L- proline Bactoagar

Table 8 – Continued

163	MS	3%	6.0	MS vitamins 3.3 mg dicamba 100 mg myo- inositol Bactoagar
171	MS	3%	6.0	MS vitamins .25 mg 2,4-D 10 mg BAP 100 mg myo- inositol Bactoagar
173	MS	6%	6.0	MS vitamins 5 mg BAP .186 mg NAA .175 mg IAA .403 mg 2IP $10^{-7}$ M ABA 200 mg myo- inositol Bactoagar
177	MS	3%	6.0	MS vitamins .25 mg 2,4-D 10 mg BAP $10^{-7}$ M ABA 100 mg myo- inositol Bactoagar
185	MS	-	5.8	3 mg BAP .04 mg NAA RT vitamins 1.65 mg thiamine 1.38 g L- proline 20 g sorbitol Bactoagar

Table 8 – Continued

10	MS	2%	6.0	.25 mg thiamine 1 mg BAP 1 mg 2,4-D 400 mg L-proline Bactoagar
19	MS	2%	6.0	.25 mg thiamine .25 mg BAP .25 mg NAA Bactoagar
20	MS	3%	6.0	.25 mg 1 mg BAP 1 mg NAA Bactoagar
52	MS	2%	6.0	.25 mg thiamine 1 mg 2,4-D $10^{-7}$ M ABA BACTOAGAR
101	MS	3%	6.0	MS vitamins 100 mg myo-inositol Bactoagar
142	MS	6%	6.0	MS vitamins 5 mg BAP 0.186 mg NAA 0.175 mg IAA 0.403 mg 2IP Bactoagar
157	MS	6%	6.0	MS vitamins 100 mg myo-inositol Bactoagar

Other possible means of identifying recipient cells include the use of isozyme markers of embryogenic cells, such as glutamate dehydrogenase, which can be detected by cytochemical stains (Fransz *et al.*, 1989). However, it is cautioned that the use of isozyme markers including glutamate dehydrogenase may lead to some degree of false positives from non-embryogenic cells such as rooty cells which nonetheless have a relatively high metabolic activity.

5 (i) *Culturing Cells to be Recipients for Transformation*

10 The inventors believe that the ability to prepare and cryopreserve cultures of maize cells is important to certain aspects of the present invention, in that it provides a means for reproducibly and successfully preparing cells for particle-mediated transformation. A variety of different types of media have been developed by the inventors and employed in carrying out various aspects of the invention. The following table, Table 8, sets forth the composition of the media preferred by the

15 inventors for carrying out these aspects of the invention.

20 **Table 8: Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Particularly Maize Cells)**

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPOUNTS** (Amount/L)
7	MS*	2%	6.0	.25 mg thiamine .5 mg BAP .5 mg NAA Bactoagar

been described in U.S. Patent No. 5,134,074; and U.S. Patent No. 5,489,520; each of which is incorporated herein by reference in its entirety.

5        Certain techniques may be used that enrich recipient cells within a cell population. For example, Type II callus development, followed by manual selection and culture of friable, embryogenic tissue, generally results in an enrichment of recipient cells for use in transformation. Suspension culturing, particularly using the media disclosed herein, may improve the ratio of recipient to non-recipient cells in any given population. Manual selection techniques which can be employed to select 10 recipient cells may include, *e.g.*, assessing cell morphology and differentiation, or may use various physical or biological means. Cryopreservation is a possible method of selecting for recipient cells.

15        Manual selection of recipient cells, *e.g.*, by selecting embryogenic cells from the surface of a Type II callus, is one means that may be used in an attempt to enrich for recipient cells prior to culturing (whether cultured on solid media or in suspension). The preferred cells may be those located at the surface of a cell cluster, and may further be identifiable by their lack of differentiation, their size and dense cytoplasm. The preferred cells will generally be those cells which are less 20 differentiated, or not yet committed to differentiation. Thus, one may wish to identify and select those cells which are cytoplasmically dense, relatively unvacuolated with a high nucleus to cytoplasm ratio (*e.g.*, determined by cytological observations), small in size (*e.g.*, 10-20  $\mu\text{m}$ ), and capable of sustained divisions and somatic proembryo formation.

25        It is proposed that other means for identifying such cells also may be employed. For example, through the use of dyes, such as Evan's blue, which are excluded by cells with relatively non-permeable membranes, such as embryogenic cells, and taken up by relatively differentiated cells such as root-like cells and snake 30 cells (so-called due to their snake-like appearance).

pollen, sperm and egg cells. It is contemplated that any cell from which a fertile plant may be regenerated is useful as a recipient cell. Type I, Type II, and Type III callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, microspores and the like. Those cells which are capable of 5 proliferating as callus also are recipient cells for genetic transformation. The present invention provides techniques for transforming immature embryos and subsequent regeneration of fertile transgenic plants. Transformation of immature embryos obviates the need for long term development of recipient cell cultures. Pollen, as well as its precursor cells, microspores, may be capable of functioning as recipient cells for 10 genetic transformation, or as vectors to carry foreign DNA for incorporation during fertilization. Direct pollen transformation, or transformation of other germline cells such as microspores or megasporangia would obviate the need for cell culture. Meristematic cells (*i.e.*, plant cells capable of continual cell division and characterized 15 by an undifferentiated cytological appearance, normally found at growing points or tissues in plants such as root tips, stem apices, lateral buds, *etc.*) may represent another type of recipient plant cell. Because of their undifferentiated growth and capacity for organ differentiation and totipotency, a single transformed meristematic cell could be recovered as a whole transformed plant. In fact, it is proposed that embryogenic suspension cultures may be an *in vitro* meristematic cell system, 20 retaining an ability for continued cell division in an undifferentiated state, controlled by the media environment.

Cultured plant cells that can serve as recipient cells for transforming with desired DNA segments may be any plant cells including corn cells, and more 25 specifically, cells from *Zea mays* L. Somatic cells are of various types. Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which typically will not respond in such a fashion. An example of non-embryogenic cells are certain Black Mexican Sweet (BMS) corn cells.

30

The development of embryogenic maize calli and suspension cultures useful in the context of the present invention, *e.g.*, as recipient cells for transformation, has

glass beads (e.g., Gene Clean™ or EluQuick™). In addition, HPLC and/or use of magnetic particles may be used to isolate DNA fragments. As an alternative to isolation of DNA fragments, a plasmid vector can be digested with a restriction enzyme and this DNA delivered to maize cells without prior purification of the 5 expression cassette fragment.

#### **IX. Recipient Cells for Transformation**

Tissue culture requires media and controlled environments. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of 10 the intact living organism. The medium usually is a suspension of various categories of ingredients (salts, amino acids, growth regulators, sugars, buffers) that are required for growth of most cell types. However, each specific cell type requires a specific range of ingredient proportions for growth, and an even more specific range of formulas for optimum growth. Rate of cell growth also will vary among cultures 15 initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding the liquid to materials capable of providing a solid support. Agar is most commonly used for this purpose. Bactoagar, Hazelton agar, Gelrite, and Gelgro are specific types of 20 solid support that are suitable for growth of plant cells in tissue culture.

Some cell types will grow and divide either in liquid suspension or on solid media. As disclosed herein, maize cells will grow in suspension or on solid medium, but regeneration of plants from suspension cultures requires transfer from liquid to 25 solid media at some point in development. The type and extent of differentiation of cells in culture will be affected not only by the type of media used and by the environment, for example, pH and light, but also by whether media is solid or liquid. Table 8 illustrates the composition of various media useful for creation of recipient cells and for plant regeneration.

30

Recipient cell targets include, but are not limited to, meristem cells, Type I, Type II, and Type III callus, immature embryos and gametic cells such as microspores,

### 1. Osmotic Adjustment

It has been suggested that osmotic pre-treatment could potentially reduce bombardment associated injury as a result of the decreased turgor pressure of the plasmolyzed cell. In a previous study, the number of cells transiently expressing GUS increased following subculture into both fresh medium and osmotically adjusted medium (U.S. Patent Serial No. 08/113,561, filed August 25, 1993, specifically incorporated herein by reference in its entirety). Pretreatment times of 90 minutes showed higher numbers of GUS expressing foci than shorter times. Cells incubated in 500 mOSM/kg medium for 90 minutes showed an approximately 3.5 fold increase in transient GUS foci than the control. Preferably, cells are precultured for 4-5 hours prior to bombardment on culture medium containing 12% sucrose. A second culture on 12% sucrose may be performed for 16-24 hours following bombardment. Alternatively, cells are pretreated on 0.2M mannitol or 12% sucrose for 3-5 hours prior to bombardment. It is contemplated that pretreatment of cells with other osmotically active solutes for a period of 1-6 hours also may be desirable.

### 2. Plasmid Configuration

In some instances, it will be desirable to deliver DNA to maize cells that does not contain DNA sequences necessary for maintenance of the plasmid vector in the bacterial host, *e.g.*, *E. coli*, such as antibiotic resistance genes, including but not limited to ampicillin, kanamycin, and tetracycline resistance, and prokaryotic origins of DNA replication. In such case, a DNA fragment containing the transforming DNA may be purified prior to transformation. An exemplary method of purification is gel electrophoresis on a 1.2% low melting temperature agarose gel, followed by recovery from the agarose gel by melting gel slices in a 6-10 fold excess of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 70°C-72°C); frozen and thawed (37°C); and the agarose pelleted by centrifugation. A Qiagen Q-100 column then may be used for purification of DNA. For efficient recovery of DNA, the flow rate of the column may be adjusted to 40 ml/hr.

30

Isolated DNA fragments can be recovered from agarose gels using a variety of electroelution techniques, enzyme digestion of the agarose, or binding of DNA to

(ii) *Biological Parameters*

Culturing conditions and other factors can influence the physiological state of the target cells and may have profound effects on transformation and integration efficiencies. First, the act of bombardment could stimulate the production of ethylene which could lead to senescence of the tissue. The addition of antiethylene compounds could increase transformation efficiencies. Second, it is proposed that certain points in the cell cycle may be more appropriate for integration of introduced DNA. Hence synchronization of cell cultures may enhance the frequency of production of transformants. For example, synchronization may be achieved using cold treatment, amino acid starvation, or other cell cycle-arresting agents. Third, the degree of tissue hydration also may contribute to the amount of trauma associated with bombardment as well as the ability of the microprojectiles to penetrate cell walls.

The position and orientation of an embryo or other target tissue relative to the particle trajectory also may be important. For example, the PDS-1000 biolistics device does not produce a uniform spread of particles over the surface of a target petri dish. The velocity of particles in the center of the plate is higher than the particle velocity at further distances from the center of the petri dish. Therefore, it is advantageous to situate target tissue on the petri dish such as to avoid the center of the dish, referred to by some as the "zone of death." Furthermore, orientation of the target tissue with regard to the trajectory of targets also can be important. It is contemplated that it is desirable to orient the tissue most likely to regenerate a plant toward the particle stream. For example, the scutellum of an immature embryo comprises the cells of greatest embryogenic potential and therefore should be oriented toward the particle stream.

It also has been reported that slightly plasmolyzed yeast cells allow increased transformation efficiencies (Armaeo *et al.*, 1990). It was hypothesized that the altered osmotic state of the cells helped to reduce trauma associated with the penetration of the microprojectile. Additionally, the growth and cell cycle stage may be important with respect to transformation.

cleavable biotinylated nucleotide chains. The DNA adheres to the particles due to the streptavidin-biotin interaction, but is released in the cell by reduction of the thiol linkage through reducing agents present in the cell.

5        Alternatively, particles may be prepared by functionalizing the surface of a gold oxide particle, providing free amine groups. DNA, having a strong negative charge, binds to the functionalized particles. Furthermore, charged particles may be deposited in controlled arrays on the surface of mylar flyer disks used in the PDS-1000 Biolistics device, thereby facilitating controlled distribution of particles  
10      delivered to target tissue.

#### 6.        Transforming DNA

As disclosed above, it further is proposed, that the concentration of DNA used to coat microprojectiles may influence the recovery of transformants containing a  
15      single copy of the transgene. For example, a lower concentration of DNA may not necessarily change the efficiency of the transformation, but may instead increase the proportion of single copy insertion events. In this regard, approximately 1 ng to 2000 ng of transforming DNA may be used per each 1.8 mg of starting microprojectiles. In other embodiments of the invention, approximately 2.5 ng to 1000 ng, 2.5 ng to 750  
20      ng, 2.5 ng to 500 ng, 2.5 ng to 250 ng, 2.5 ng to 100 ng, or 2.5 ng to 50 ng of transforming DNA may be used per each 1.8 mg of starting microprojectiles.

Various other methods also may be used to increase transformation efficiency and/or increase the relative proportion of low-copy transformation events. For  
25      example, the inventors contemplate end-modifying transforming DNA with alkaline phosphatase or an agent which will blunt DNA ends prior to transformation. Still further, an inert carrier DNA may be included with the transforming DNA, thereby lowering the effective transforming DNA concentration without lowering the overall amount of DNA used. These techniques are described in further detail in U.S. Patent  
30      Ser. No. 08/995,451, filed December 22, 1997, the disclosure of which is specifically incorporated herein by reference in its entirety.

#### 4. Helium Pressure

By manipulation of the type and number of rupture disks, pressure can be varied between 400 and 2000 psi within the gas acceleration tube. Optimum pressure for stable transformation has been determined to be between 1000 and 1200 psi.

5

#### 5. Coating of Microprojectiles.

For microprojectile bombardment, one will attach (*i.e.*, "coat") DNA to the microprojectiles such that it is delivered to recipient cells in a form suitable for transformation thereof. In this respect, at least some of the transforming DNA must be available to the target cell for transformation to occur, while at the same time during delivery the DNA must be attached to the microprojectile. Therefore, availability of the transforming DNA from the microprojectile may comprise the physical reversal of interactions between transforming DNA and the microprojectile following delivery of the microprojectile to the target cell. This need not be the case, however, as availability to a target cell may occur as a result of breakage of unbound segments of DNA or of other molecules which comprise the physical attachment to the microprojectile. Availability may further occur as a result of breakage of bonds between the transforming DNA and other molecules, which are either directly or indirectly attached to the microprojectile. It is further contemplated that transformation of a target cell may occur by way of direct illegitimate or homology-dependent recombination between the transforming DNA and the genomic DNA of the recipient cell. Therefore, as used herein, a "coated" microprojectile will be one which is capable of being used to transform a target cell, in that the transforming DNA will be delivered to the target cell, yet will be accessible to the target cell such that transformation may occur.

Any technique for coating microprojectiles which allows for delivery of transforming DNA to the target cells may be used. Methods for coating microprojectiles which have been demonstrated to work well with the current invention have been specifically disclosed herein. DNA may be bound to microprojectile particles using alternative techniques, however. For example, particles may be coated with streptavidin and DNA end labeled with long chain thiol

bombardment parameters, and the plasmid configuration have been adjusted to yield the maximum numbers of stable transformants.

5 (i) *Physical Parameters*

1. Gap Distance

The variable nest (macro holder) can be adjusted to vary the distance between the rupture disk and the macroprojectile, *i.e.*, the gap distance. This distance can be varied from 0 to 2 cm. The predicted effects of a shorter gap are an increase of velocity of both the macro- and microprojectiles, an increased shock wave (which leads to tissue splattering and increased tissue trauma), and deeper penetration of microprojectiles. Longer gap distances would have the opposite effects but may increase viability and therefore the total number of recovered stable transformants.

15 2. Flight Distance

The fixed nest (contained within the variable nest) can be varied between 0.5 and 2.25 cm in predetermined 0.5 cm increments by the placement of spacer rings to adjust the flight path traversed by the macroprojectile. Short flight paths allow for greater stability of the macroprojectile in flight but reduce the overall velocity of the microprojectiles. Increased stability in flight increases, for example, the number of 20 centered GUS foci. Greater flight distances (up to some point) increase velocity but also increase instability in flight. Based on observations, it is recommended that bombardments typically be done with a flight path length of about 1.0 cm to 1.5 cm.

3. Tissue Distance

25 Placement of tissue within the gun chamber can have significant effects on microprojectile penetration. Increasing the flight path of the microprojectiles will decrease velocity and trauma associated with the shock wave. A decrease in velocity also will result in shallower penetration of the microprojectiles.

### VIII. Optimization of Microprojectile Bombardment

For microprojectile bombardment transformation using the constructs of the instant invention, both physical and biological parameters may be optimized. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or 5 those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, such as the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, the orientation of an immature embryo or other target tissue relative to the particle trajectory, and also the nature of 10 the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

Accordingly, it is contemplated that one may wish to adjust various 15 bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as DNA concentration, gap distance, flight distance, tissue distance, and helium pressure. It is further contemplated that the grade of helium may effect transformation efficiency. For example, differences in transformation efficiencies may be witnessed between 20 bombardments using industrial grade (99.99% pure) or ultra pure helium (99.999% pure), although it is not currently clear which is more advantageous for use in bombardment. One also may optimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For 25 example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation.

Other physical factors include those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either 30 the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells immediately before and after bombardment. The prebombardment culturing conditions, such as osmotic environment, the

*Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

5 (iv) *Other Transformation Methods*

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, e.g., Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Omirulleh *et al.*, 1993; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

10

Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts have been described (Fujimara *et al.*, 1985; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986; Omirulleh *et al.*, 15 1993 and U.S. Patent No. 5,508,184; each specifically incorporated herein by reference in its entirety). Examples of the use of direct uptake transformation of cereal protoplasts include transformation of rice (Ghosh-Biswas *et al.*, 1994), sorghum (Battraw and Hall, 1991), barley (Lazerri, 1995), oat (Zheng and Edwards, 1990) and maize (Omirulleh *et al.*, 1993).

20

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1989). Also, silicon carbide fiber-mediated transformation may be used with or without protoplasting (Kaepller, 1990; Kaepller *et al.*, 1992; U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety). Transformation with this technique is accomplished by agitating silicon carbide fibers together with cells in a DNA solution. DNA passively enters as the cell are punctured. This technique has been used successfully with, for example, 25 the monocot cereals maize (PCT Application WO 95/06128, specifically incorporated herein by reference in its entirety; Thompson, 1995) and rice (Nagatani, 1997).

(iii) *Agrobacterium-mediated Transformation*

5 *Agrobacterium*-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described by Fraley *et al.*, (1985), Rogers *et al.*, (1987) and U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety.

10 *Agrobacterium*-mediated transformation is most efficient in dicotyledonous plants and is the preferable method for transformation of dicots, including *Arabidopsis*, tobacco, tomato, and potato. Indeed, while *Agrobacterium*-mediated transformation has been routinely used with dicotyledonous plants for a number of years, it has only recently become applicable to monocotyledonous plants. Advances 15 in *Agrobacterium*-mediated transformation techniques have now made the technique applicable to nearly all monocotyledonous plants. For example, *Agrobacterium*-mediated transformation techniques have now been applied to rice (Hiei *et al.*, 1997; Zhang *et al.*, 1997; U.S. Patent No. 5,591,616, specifically incorporated herein by reference in its entirety), wheat (McCormac *et al.*, 1998), barley (Tingay *et al.*, 1997; 20 McCormac *et al.*, 1998), and maize (Ishidia *et al.*, 1996).

25 Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate the construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987) have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are 30 suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant strains where

Hence, it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with monocot plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

Microprojectile bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species for which have been transformed by microprojectile bombardment include monocot species such as maize (PCT Application WO 95/06128), barley (Ritala *et al.*, 1994; Hensgens *et al.*, 1993), wheat (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety), rice (Hensgens *et al.*, 1993), oat (Torbet *et al.*, 1995; Torbet *et al.*, 1998), rye (Hensgens *et al.*, 1993), sugarcane (Bower *et al.*, 1992), and sorghum (Casa *et al.*, 1993; Hagio *et al.*, 1991); as well as a number of dicots including tobacco (Tomes *et al.*, 1990; Busing and Benbow, 1994), soybean (U.S. Patent No. 5,322,783, specifically incorporated herein by reference in its entirety), sunflower (Knittel *et al.* 1994), peanut (Singsit *et al.*, 1997), cotton (McCabe and Martinell, 1993), tomato (VanEck *et al.* 1995), and legumes in general (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety).

To effect transformation by electroporation, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this 5 technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Patent No. 5,384,253; Rhodes *et al.*, 1995; D'Halluin, 1992), wheat (Zhou *et al.*, 1993), tomato (Hou and Lin, 1996), 10 soybean (Christou *et al.*, 1987) and tobacco (Lee *et al.*, 1989).

One also may employ protoplasts for electroporation transformation of plants (Bates, 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and 15 Widholm in Intl. Patent Appl. Publ. No. WO 9217598 (specifically incorporated herein by reference). Other examples of species for which protoplast transformation has been described include barley (Lazzeri, 1995), sorghum (Battraw *et al.*, 1991), maize (Bhattacharjee *et al.*, 1997), wheat (He *et al.*, 1994) and tomato (Tsukada, 1989).

20

(ii) *Microprojectile Bombardment*

A preferred method for delivering transforming DNA segments to plant cells in accordance with the invention is microprojectile bombardment (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 5,610, 042; and PCT Application WO 94/09699; each of which is specifically incorporated herein by reference in its entirety). In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA 25 delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. 30

can be positioned around an expressible gene of interest to effect an increase in overall expression of the gene and diminish position dependent effects upon incorporation into the plant genome (Stief *et al.*, 1989; Phi-Van *et al.*, 1990).

5        **VII. Transformation**

There are many methods for transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell, such as by direct delivery of DNA such as by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), by electroporation (U.S. Patent No. 5,384,253, specifically incorporated herein by reference in its entirety), by agitation with silicon carbide fibers (Kaepller *et al.*, 1990; U.S. Patent No. 5,302,523, specifically incorporated herein by reference in its entirety; and U.S. Patent No. 5,464,765, specifically incorporated herein by reference in its entirety), by *Agrobacterium*-mediated transformation (U.S. Patent No. 5,591,616 and U.S. Patent No. 5,563,055; both specifically incorporated herein by reference) and by acceleration of DNA coated particles (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,877; and U.S. Patent No. 5,538,880; each specifically incorporated herein by reference in its entirety), *etc.* Through the application of techniques such as these, maize cells as well as those of virtually any other plant species may be stably transformed, and these cells developed into transgenic plants. In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

25            (i)        *Electroporation*

Where one wishes to introduce DNA by means of electroporation, it is contemplated that the method of Krzyzek *et al.* (U.S. Patent No. 5,384,253, incorporated herein by reference in its entirety) will be particularly advantageous. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells are made more susceptible to transformation by mechanical wounding.

gene may encode all or part of the targeted native protein but its translation may not be required for reduction of levels of that native protein.

## 2. Non-RNA-Expressing

5 DNA elements including those of transposable elements such as Ds, Ac, or Mu, may be inserted into a gene to cause mutations. These DNA elements may be inserted in order to inactivate (or activate) a gene and thereby "tag" a particular trait. In this instance the transposable element does not cause instability of the tagged mutation, because the utility of the element does not depend on its ability to move in 10 the genome. Once a desired trait is tagged, the introduced DNA sequence may be used to clone the corresponding gene, *e.g.*, using the introduced DNA sequence as a PCR primer together with PCR gene cloning techniques (Shapiro, 1983; Dellaporta *et al.*, 1988). Once identified, the entire gene(s) for the particular trait, including control or regulatory regions where desired, may be isolated, cloned and manipulated as 15 desired. The utility of DNA elements introduced into an organism for purposes of gene tagging is independent of the DNA sequence and does not depend on any biological activity of the DNA sequence, *i.e.*, transcription into RNA or translation into protein. The sole function of the DNA element is to disrupt the DNA sequence of a gene.

20

It is contemplated that unexpressed DNA sequences, including novel synthetic sequences, could be introduced into cells as proprietary "labels" of those cells and plants and seeds thereof. It would not be necessary for a label DNA element to disrupt the function of a gene endogenous to the host organism, as the sole function of 25 this DNA would be to identify the origin of the organism. For example, one could introduce a unique DNA sequence into a plant and this DNA element would identify all cells, plants, and progeny of these cells as having arisen from that labeled source. It is proposed that inclusion of label DNAs would enable one to distinguish proprietary germplasm or germplasm derived from such, from unlabelled germplasm.

30

Another possible element which may be introduced is a matrix attachment region element (MAR), such as the chicken lysozyme A element (Stief, 1989), which

Genes may be constructed or isolated, which when transcribed, produce antisense RNA that is complementary to all or part(s) of a targeted messenger RNA(s). The antisense RNA reduces production of the polypeptide product of the messenger RNA. The polypeptide product may be any protein encoded by the plant genome. The aforementioned genes will be referred to as antisense genes. An antisense gene may thus be introduced into a plant by transformation methods to produce a novel transgenic plant with reduced expression of a selected protein of interest. For example, the protein may be an enzyme that catalyzes a reaction in the plant. Reduction of the enzyme activity may reduce or eliminate products of the reaction which include any enzymatically synthesized compound in the plant such as fatty acids, amino acids, carbohydrates, nucleic acids and the like. Alternatively, the protein may be a storage protein, such as a zein, or a structural protein, the decreased expression of which may lead to changes in seed amino acid composition or plant morphological changes respectively. The possibilities cited above are provided only by way of example and do not represent the full range of applications.

Genes also may be constructed or isolated, which when transcribed produce RNA enzymes, or ribozymes, which can act as endoribonucleases and catalyze the cleavage of RNA molecules with selected sequences. The cleavage of selected messenger RNAs can result in the reduced production of their encoded polypeptide products. These genes may be used to prepare novel transgenic plants which possess them. The transgenic plants may possess reduced levels of polypeptides including, but not limited to, the polypeptides cited above that may be affected by antisense RNA.

25

It also is possible that genes may be introduced to produce novel transgenic plants which have reduced expression of a native gene product by a mechanism of co-suppression. It has been demonstrated in tobacco, tomato, and petunia (Goring *et al.*, 1991; Smith *et al.*, 1990; Napoli *et al.*, 1990; van der Krol *et al.*, 1990) that expression of the sense transcript of a native gene will reduce or eliminate expression of the native gene in a manner similar to that observed for antisense genes. The introduced

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CLAIMS:

1. A chimeric gene comprising an aleurone specific promoter operably linked to a gene encoding a screenable marker.
- 5 2. The chimeric gene of claim 1, wherein said screenable marker is selected from the group consisting of GFP, luciferase and an R gene.
- 10 3. The chimeric gene of claim 1, wherein said promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine carboxypeptidaseII promoter, catalase promoter, alpha-glucosidase promoter, beta-amylase promoter, VP1 promoter, and bronze2 promoter.
- 15 4. The chimeric gene of claim 1, wherein said promoter is an L3 oleosin promoter.
- 20 5. A construct comprising a gene fusion between a selectable marker gene and a screenable marker gene, wherein said gene fusion is operably linked to an aleurone-specific promoter.
6. The construct of claim 5, wherein said screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene.
- 25 7. The construct of claim 6, wherein said selectable marker gene comprises a gene selected from the group consisting of NPTII, bar, EPSPS, anthranilate synthase and dalapon dehalogenase.
- 30 8. The construct of claim 5, wherein said promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine carboxypeptidaseII

promoter, catalase promoter, alpha-glucosidase promoter, beta-amylase promoter, VP1 promoter, and bronze2 promoter.

5 9. The construct of claim 8, wherein said oleosin promoter is an L3 oleosin promoter.

10 10. The construct of claim 5, further comprising a second gene.

11. The construct of claim 10, wherein said second gene is selected from the group 10 consisting of an insect resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene 15 affecting plant agronomic characteristics, and an environment or stress resistance gene.

12. The construct of claim 11, wherein said second gene is operably linked to a 20 second promoter.

13. A transgenic plant comprising the chimeric gene of claim 1.

14. The transgenic plant of claim 13, wherein said screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene.

25 15. The transgenic plant of claim 13, wherein said promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine 30 carboxypeptidaseII promoter, catalase promoter, alpha-glucosidase promoter, beta-amylase promoter, VP1 promoter, and bronze2 promoter.

16. The transgenic plant of claim 13, wherein said promoter is an L3 oleosin promoter.

17. The transgenic plant of claim 13, further defined as a monocotyledonous plant.

5 18. The transgenic plant of claim 17, wherein said monocotyledonous plant is further defined as a plant selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, sugarcane and turfgrass.

10 19. The transgenic plant of claim 18, wherein said monocotyledonous plant is maize.

20. The transgenic plant of claim 13, further defined as a dicotyledonous plant.

15 21. The transgenic plant of claim 20, wherein said dicotyledonous plant is selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

22. The transgenic plant of claim 21, wherein the dicotyledonous plant is soybean.

20 23. A progeny plant of any generation of the plant of claim 13, wherein said progeny plant comprises said chimeric gene.

25 24. A transgenic plant comprising a gene fusion between a selectable marker gene and a screenable marker gene, wherein said gene fusion is operably linked to an aleurone-specific promoter.

25. The transgenic plant of claim 24, wherein said screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene.

30 26. The transgenic plant of claim 25, wherein said screenable marker is a GFP gene.

27. The transgenic plant of claim 24, wherein said selectable marker gene is selected from the group consisting of NPTII, bar, EPSPS, anthranilate synthase and dalapon dehalogenase.

5 28. The transgenic plant of claim 24, wherein said aleurone-specific promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine carboxypeptidaseII promoter, catalase promoter, alpha-glucosidase promoter, 10 beta-amylase promoter, VP1 promoter, and bronze2 promoter.

29. The transgenic plant of claim 28, wherein said oleosin promoter is an L3 oleosin promoter.

15 30. The transgenic plant of claim 24, further defined as a monocotyledonous plant.

31. The transgenic plant of claim 30, wherein said monocotyledonous plant is selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, sugarcane and turfgrass.

20 32. The transgenic plant claim 31, wherein said monocotyledonous plant is maize.

33. The transgenic plant of claim 24, further defined as a dicotyledonous plant.

25 34. The transgenic plant of claim 33, wherein said dicotyledonous plant is selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

35. The plant of claim 34, wherein the dicotyledonous plant is soybean.

30 36. A progeny plant of any generation of the transgenic plant of claim 24, wherein said progeny plant comprises said gene fusion.

37. A method of identifying transgenic seeds comprising the steps of:

- (a) obtaining a transgenic plant, the cells of which comprise a chimeric gene comprising an aleurone specific promoter operably linked to a gene encoding a screenable marker;
- 5 (b) cultivating said transgenic plant until seed set;
- (c) collecting seeds produced on said transgenic plant; and
- (d) screening said seeds based on a phenotype conferred upon the seeds by said screenable marker gene.

10 38. The method of claim 37, wherein said screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene.

15 39. The method of claim 38, wherein said screenable marker is a GFP gene.

40. The method of claim 37, wherein said transgenic plant is a monocotyledonous plant.

18 41. The method of claim 40, wherein said monocotyledonous plant is selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, 20 sugarcane and turfgrass.

42. The method of claim 41, wherein said monocotyledonous plant is maize.

43. The method of claim 37, wherein said transgenic plant is a dicotyledonous plant.

25 44. The method of claim 43, wherein said dicotyledonous plant is selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

30 45. The method of claim 44, wherein the dicotyledonous plant is soybean.

46. The method of claim 37, wherein said plant further comprises a second gene encoding a selected trait, wherein said exogenous gene is genetically linked to said chimeric gene.

5 47. The method of claim 46, wherein said second gene comprises a second promoter operatively linked to said second gene.

10 48. The method of claim 47, wherein said second gene is selected from the group consisting of an insect resistance gene, a viral disease resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a nematode disease resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, 15 and an environment or stress resistance gene.

49. The method of claim 37, wherein said chimeric gene is operably linked to a 3' region.

20 50. The method of claim 37, wherein said chimeric gene comprises an element enhancing the expression of said chimeric gene.

51. The method of claim 37, wherein said screening is automated.

25 52. A method of identifying transgenic seeds comprising the steps of:  
(a) obtaining a transgenic plant, the cells of which comprise a gene fusion between a selectable marker gene and a screenable marker gene, wherein said gene fusion is operably linked to an aleurone-specific promoter;

30 (b) cultivating said transgenic plant until seed set;  
(c) collecting seeds produced on said transgenic plant; and

(d) screening said seeds based on a phenotype conferred upon the seeds by said screenable marker gene.

53. The method of claim 52, wherein said screenable marker gene is selected from  
5 the group consisting of a GFP gene, a luciferase gene, and an R gene.

54. The method of claim 53, wherein said screenable marker is a GFP gene.

55. The method of claim 52, wherein said transgenic plant is a monocotyledonous  
10 plant.

56. The method of claim 55, wherein said monocotyledonous plant is selected  
from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum,  
sugarcane and turfgrass.

15

57. The method of claim 56, wherein said monocotyledonous plant is maize.

58. The method of claim 52, wherein said transgenic plant is a dicotyledonous  
plant.

20

59. The method of claim 58, wherein said dicotyledonous plant is selected from  
the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

60. The method of claim 59, wherein the dicotyledonous plant is soybean.

25

61. The method of claim 52, wherein said transgenic plant further comprises an  
exogenous gene encoding a selected trait, wherein said exogenous gene is genetically  
linked to said gene fusion.

30

62. The method of claim 61, wherein said exogenous gene comprises a second  
promoter operatively linked to said exogenous gene.

63. The method of claim 61, wherein said exogenous gene encodes a trait selected from the group consisting of an insect resistance gene, a viral disease resistance gene, a bacterial disease resistance gene, a fungal disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a nematode disease resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a gene affecting plant agronomic characteristics, and an environment or stress resistance gene.

10 64. The method of claim 52, wherein said gene fusion comprises a 3' region operatively linked to said gene fusion.

65. The method of claim 52, wherein said gene fusion comprises an element enhancing the expression of said gene fusion.

15 66. The method of claim 52, wherein said screening is automated.

67. A method of plant breeding comprising the steps of:

20 (a) obtaining first and second plants, wherein cells of one or both of said first or second plants comprise a screenable marker gene operably linked to an aleurone-specific promoter;  
(b) crossing said first and second plants; and  
(c) collecting the seeds resulting from said crossing.

25 68. The method of claim 67, further comprising the step of:

(d) selecting transgenic seeds from collected seeds based on a phenotype conferred upon said transgenic seeds by said screenable marker gene.

30 69. The method of claim 67, wherein said screenable marker gene is fused to a selectable marker.

70. The method of claim 67, wherein said screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene.

71. The method of claim 70, wherein said screenable marker is a GFP gene.

5

72. The method of claim 69, wherein said selectable marker gene comprises a gene selected from the group consisting of NPTII, bar, EPSPS, anthranilate synthase and dalapon dehalogenase.

10 73. The method of claim 67, wherein said promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine carboxypeptidaseII promoter, catalase promoter, alpha-glucosidase promoter, beta-amylase promoter,  
15 VP1 promoter, and bronze2 promoter.

74. The method of claim 73, wherein said promoter is an oleosin promoter.

20 75. The method of claim 74, wherein said oleosin promoter is an L3 oleosin promoter.

76. The method of claim 73, wherein said promoter is a globulin 1 promoter.

77. The method of claim 73, wherein said promoter is a barley LTP2 promoter.

25

78. The method of claim 67, wherein said first and second transgenic plants are monocotyledonous plants.

30

79. The method of claim 78, wherein said monocotyledonous plants are selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, sugarcane and turfgrass.

80. The method of claim 79, wherein said monocotyledonous plants are maize plants.

5 81. The method of claim 67, wherein said first and second transgenic plants are dicotyledonous plants.

82. The method of claim 81, wherein said dicotyledonous plants are selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

10 83. The method of claim 82, wherein the dicotyledonous plants are soybean plants.

84. The method of claim 68, wherein said selecting is automated.

85. A method of plant breeding comprising the steps of:

15 (a) obtaining a transgenic plant, the cells of which comprise a screenable marker gene operably linked to an aleurone-specific promoter;  
(b) allowing said plant to self fertilize; and  
(c) collecting the seeds produced on said plant.

20 86. The method of claim 85, further comprising the step of:

(d) selecting transgenic seeds from said collected seeds based on the phenotype conferred upon said transgenic seeds by said screenable marker gene.

25 87. The method of claim 85, wherein said screenable marker gene is fused to a selectable marker gene.

88. The method of claim 85, wherein said screenable marker gene is selected from the group consisting of a GFP gene, a luciferase gene, and an R gene.

30

89. The method of claim 88, wherein said screenable marker is a GFP gene.

90. The method of claim 87, wherein said selectable marker gene comprises a gene selected from the group consisting of NPTII, bar, EPSPS, anthranilate synthase and dalapon dehalogenase.

5 91. The method of claim 85, wherein said promoter is selected from the group consisting of an oleosin promoter, globulin 1 promoter, barley LTP2 promoter, alpha-amylase promoter, chitinase promoter, beta-glucanase promoter, cysteine proteinase promoter, glutaredoxin promoter, HVA1 promoter, serine carboxypeptidaseII promoter, catalase promoter, alpha-glucosidase promoter, beta-amylase promoter,  
10 VP1 promoter, and bronze2 promoter.

92. The method of claim 91, wherein said oleosin promoter is an L3 oleosin promoter.

15 93. The method of claim 91, wherein said promoter is a globulin 1 promoter.

94. The method of claim 91, wherein said promoter is a barley LTP2 promoter.

95. The method of claim 85, wherein said transgenic plant is a monocotyledonous  
20 plant.

96. The method of claim 95, wherein said monocotyledonous plant is selected from the group consisting of maize, rice, wheat, barley, oat, rye, millet, sorghum, sugarcane and turfgrass.

25

97. The method of claim 96, wherein said monocotyledonous plant is a maize plant.

98. The method of claim 85, wherein said transgenic plant is a dicotyledonous  
30 plant.

99. The method of claim 98, wherein said dicotyledonous plant is selected from the group consisting of cotton, soybean, tomato, potato, citrus, and tobacco.

100. The method of claim 99, wherein the dicotyledonous plant is a soybean plant.

5

101. The method of claim 86, wherein said step of selecting is automated.

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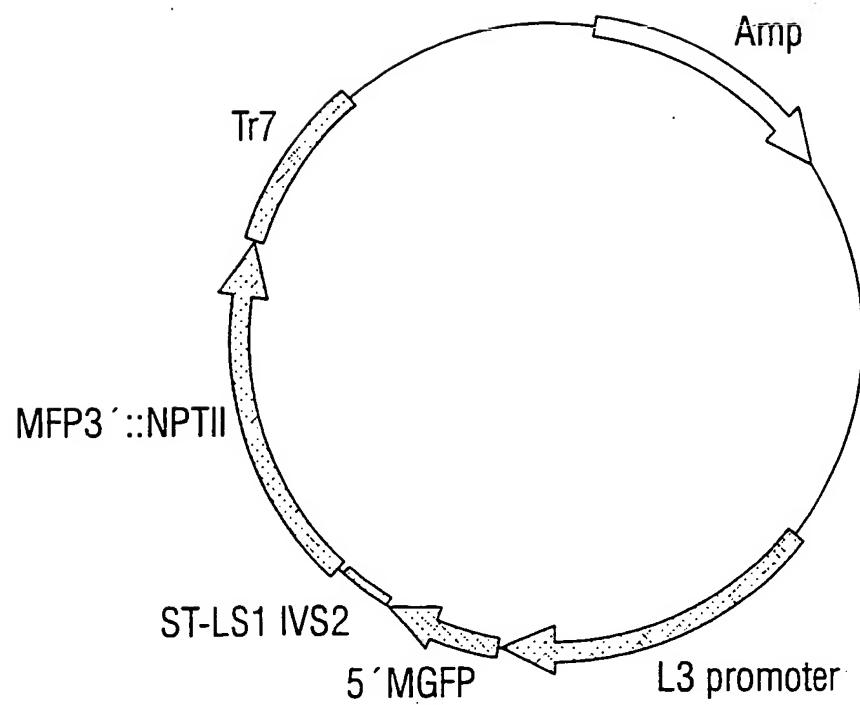


FIG. 1

2/6

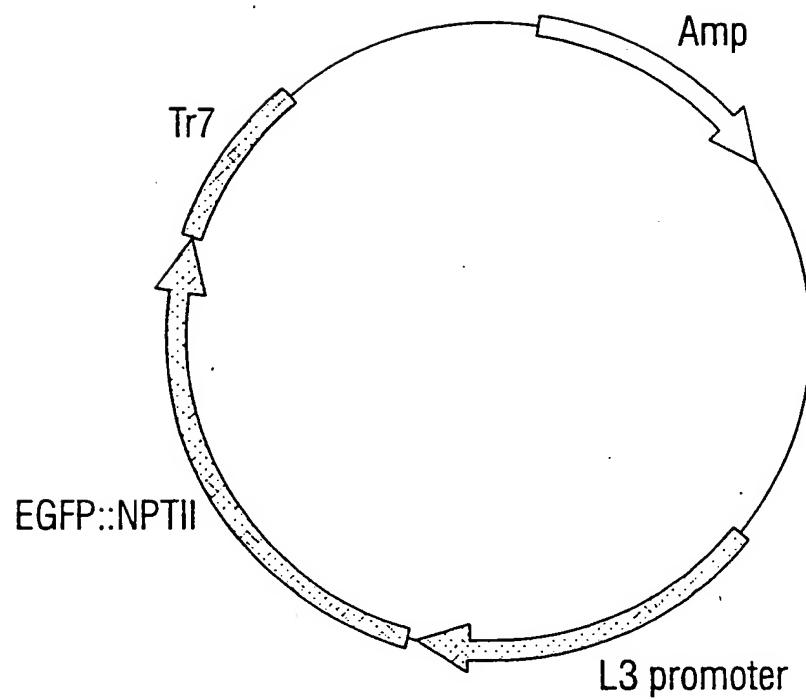


FIG. 2

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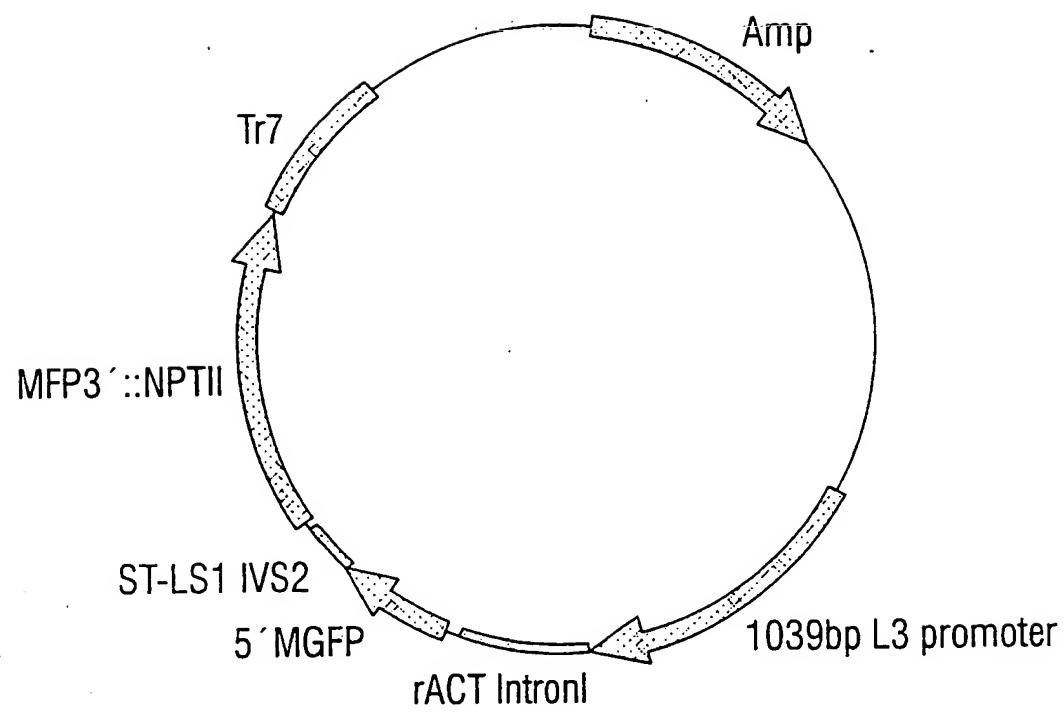


FIG. 3

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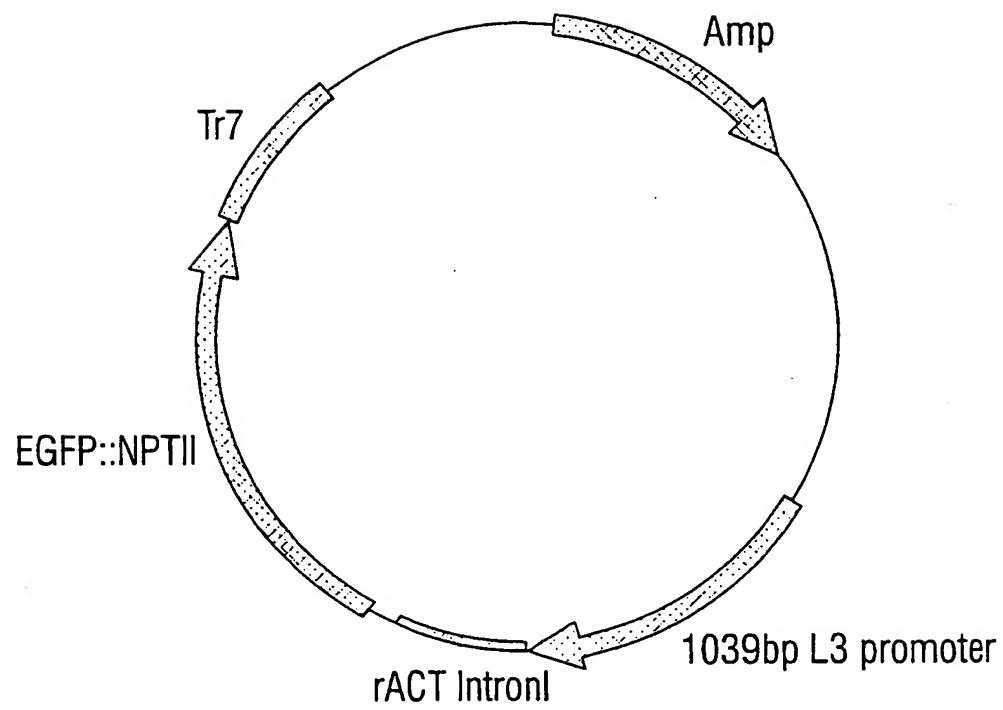


FIG. 4

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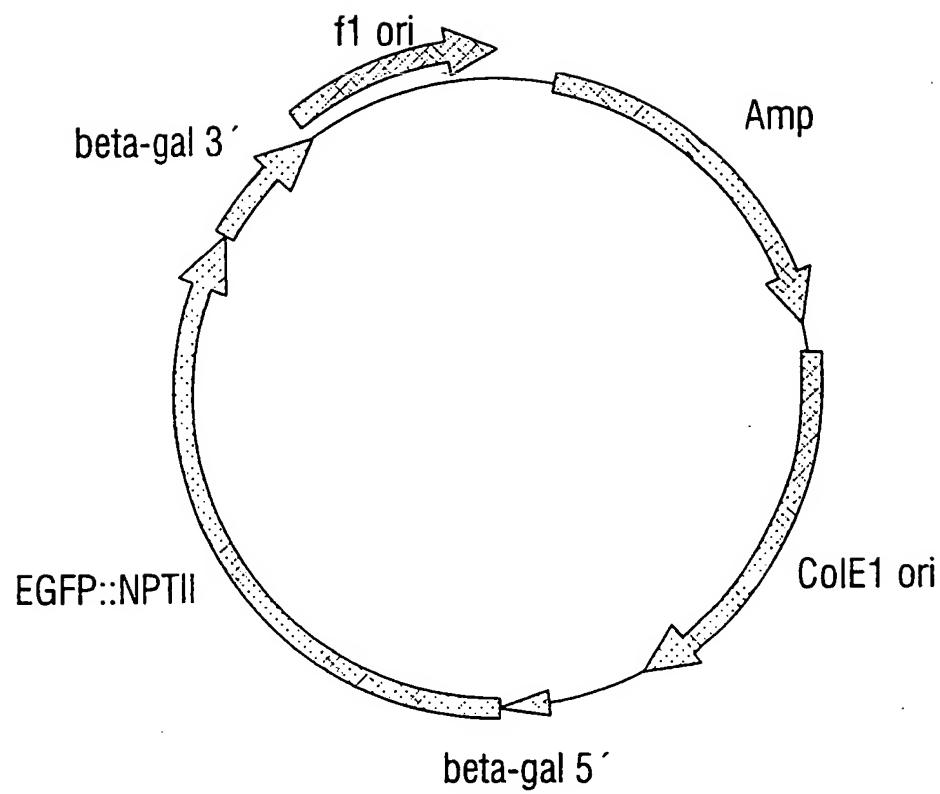


FIG. 5

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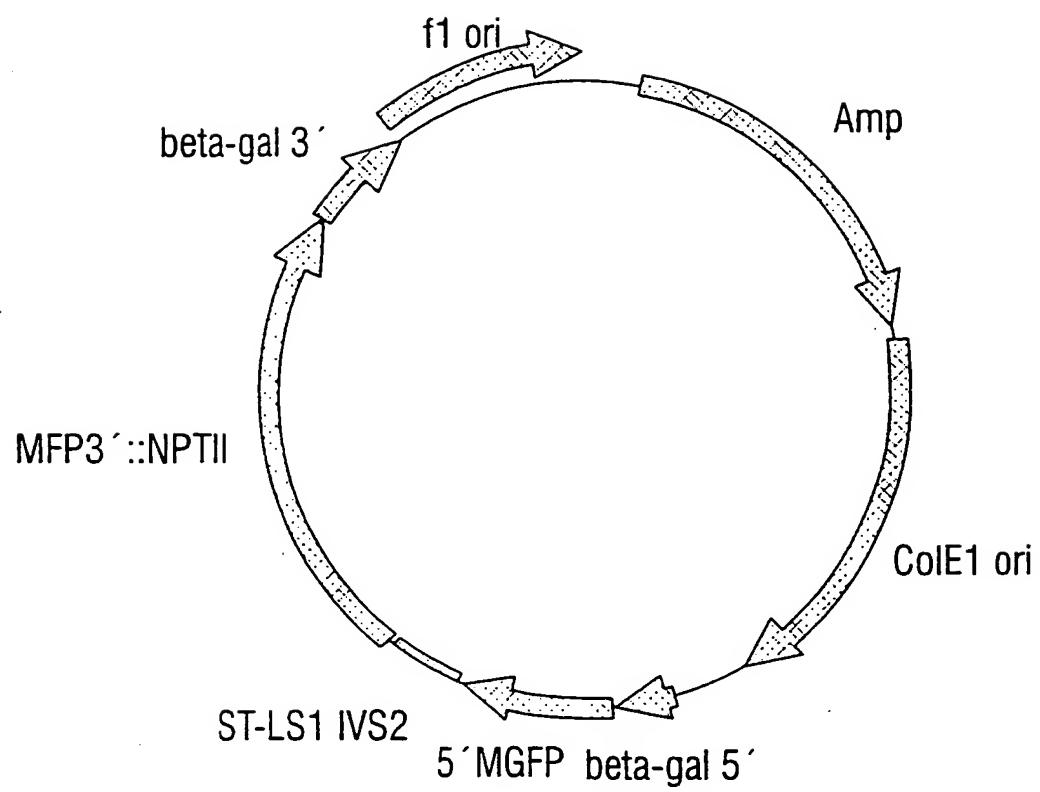


FIG. 6

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26

## INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 99/11023

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C12N15/29 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 23230 A (KALLA ROGER ; OLSEN ODD ARNE (NO); LINNESTAD CASPER (NO)) 31 August 1995 (1995-08-31) the whole document ----	1-3, 13-15, 17-23
X	WO 95 34634 A (LEEMANS JAN ; WILLIAMS MARK (BE); PLANT GENETIC SYSTEMS NV (BE); KR) 21 December 1995 (1995-12-21)	1-4, 13-23, 37-51, 67,68, 70,71, 73-86, 88,89, 91-101
Y	see esp. p.38 1.22 - p-42; examples ----	5-12, 24-36, 52-66, 69,72,87 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

6 September 1999

Date of mailing of the international search report

17/09/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
 Fax: (+31-70) 340-3016

Authorized officer

Kania, T

## INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/11023

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 17593 A (CANADA NAT RES COUNCIL) 15 October 1992 (1992-10-15)  the whole document ---	5-12, 24-36, 52-66, 69,72,87
A	LEE K. AND HUANG A.: "Genes encoding oleosins in maize kernel of inbreds Mo17 and B73" PLANT MOLECULAR BIOLOGY, vol. 26, 1994, pages 1981-1987, XP002104492 cited in the application the whole document ---	4,9,16, 29,75,92
A	WO 97 41228 A (PIONEER HI BRED INT ;GORDON KAMM WILLIAM (US); PIERCE DORTOTHY A () 6 November 1997 (1997-11-06) cited in the application the whole document ---	1-101
A	US 5 474 929 A (PELCHER LAWRENCE E) 12 December 1995 (1995-12-12) the whole document ---	1-101
A	WO 91 02059 A (PIONEER HI BRED INT) 21 February 1991 (1991-02-21) the whole document ---	1-101
A	BENNETT R P ET AL: "Fusion of green fluorescent protein with the Zeocin-resistance marker allows visual screening and drug selection of transfected eukaryotic cells." BIOTECHNIQUES, (1998 MAR) 24 (3) 478-82. , XP002114401 the whole document -----	1-101

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/11023

## Box I Observations where certain claims were found uns archabl (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
See FURTHER INFORMATION sheet PCT/ISA/210
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

As claims 67-101 are directed to general methods of plant breeding that are not patentable in the light of Rule 39 PCT, the search was performed only with respect to the plants used in said methods.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

**INTERNATIONAL SEARCH REPORT**

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